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Graduate in Biochemistry, PhD in Biochemistry

Combining Vermifiltration with Hydroponics to Treat Organic Wastewaters and Produce Food

Thesis submitted for the Degree of Master of Biotechnology

Supervisor: Doctor Luís Miguel Igreja Aires,
Professor Adjunto, Escola Superior de Tecnologia e Gestão, Politécnico de Leiria

Co-supervisor: Doctor Nídia Dana Lourenço,
Researcher, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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February 2021

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POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TECNOLOGIA E GESTÃO
U.PORTO FEUP FACULDADE DE ENGENHARIA UNIVERSIDADE DO PORTO
SRE CM ASSOCIATE LABORATORY LABORATORY OF SEPARATION AND REACTION ENGINEERING LABORATORY OF CATALYSIS AND MATERIALS
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Resumo

A carne de porco é consumida no Mundo inteiro. A suinicultura em larga escala satisfaz a procura do mercado, mas gera grandes quantidades de resíduos ricos em bactérias patogénicas, matéria orgânica, azoto e fósforo. O seu tratamento habitual é insuficiente para eliminar o risco de poluição. A vermicompostagem e a vermifiltração têm demonstrado a capacidade de remover matéria orgânica, controlar organismos patogénicos e promover a oxidação de amónia e nitrito em resíduos pecuários sólidos e líquidos. Plantas em hidroponia são capazes de remover nitratos e fósforo, diminuindo o risco de eutrofização.

Foi construído um sistema parcialmente contínuo em que se combinou um vermifiltro com uma unidade de hidroponia em água profunda a jusante. Matéria orgânica, microrganismos patogénicos fecais e formas de azoto foram analisadas na etapa de vermifiltração; a análise da hidroponia foi focada no azoto e no fósforo. Comunidades bacterianas nitrificantes foram analisadas em vários pontos de amostragem. Foi apresentada uma reflexão sobre a possibilidade de escalar o sistema para condições reais.

A vermifiltração removeu amónia (≤100%), nitrito (≤100%), matéria orgânica (≤83% BOD_5) e coliformes (≤54%). Na hidroponia, couve-coração e chicória tiveram um crescimento inicial, posteriormente observando-se carências nutricionais, paragem do crescimento e morte. AOB e NOB foram observadas em abundância moderada na fase líquida, sendo necessários mais estudos em sólidos e biofilmes. O tratamento hidropónico do efluente do vermifiltro removeu BOD_5 (≤83%), amónia (≤98%), nitrito (≤99%) e fósforo (≤55%), sendo limitado provavelmente por fatores ecológicos. Os nitratos não foram significativamente removidos no tratamento hidropónico, e a população de coliformes recuperou. Concluiu-se que é necessário monitorizar e corrigir condições físicas e nutricionais e controlar microrganismos no sistema. Estudos preliminares deverão ser feitos em *batch*. É possível implementar sistemas semelhantes em suiniculturas, ainda que tal requeira investir na construção, controlo, manutenção e pessoal.

Termos chave: águas residuais suinícolas, tratamento biológico, vermifiltração, culturas hidropónicas, recirculação do efluente.

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Abstract

Pork is extensively consumed worldwide. Large-scale pig farming responds to the consumer demand but also generates great amounts of waste, rich in pathogenic bacteria, organic matter, nitrogen and phosphorus. The typical piggery waste treatment is insufficient to eliminate the risk of pollution. Vermicomposting and vermifiltration have shown capacity to remove organic matter, control pathogens and promote ammonia and nitrite oxidation in animal wastes and wastewaters. Hydroponic plants have shown ability to remove nitrate and phosphorus from wastewaters and decrease eutrophication risks.

A partially continuous pilot-scale system combining a trickling vermifilter with a downstream hydroponic deep-water culture unit was built. Organic matter, faecal pathogens, and forms of nitrogen were analysed in the vermifiltration stage; nitrogen and phosphorus were the focus of hydroponic treatment analysis. Nitrifying bacterial communities were analysed in different sampling spots in the system. A reflection on the possibility to upscale the system for real-life use was presented.

Vermifiltration removed ammonia (≤100%), nitrite (≤100%), organic matter (≤83% BOD_5), and coliform bacteria (≤54%). A variety of pointed cabbage and radicchio initially grew hydroponically but showed signs of nutrient deficiencies, stalled growth and death over time. Modest abundances of AOB and NOB were found in the liquid phase of the system, and further study of solids and biofilms are needed. Hydroponic treatment of the vermifilter effluent removed more BOD_5 (≤83%), ammonia (≤98%), nitrite (≤99%) and phosphorus (≤55%), removal limited probably by ecological factors. Nitrate was not efficiently removed by hydroponic treatment. Coliforms recovered in the hydroponic unit. The results suggest the need to monitor and correct physical conditions and nutrient content in the feed, and to control the micro-organisms throughout the system. Batch systems are advisable for preliminary studies. Implementation of similar systems on pig farms facilities is possible, although requiring investment in construction, monitoring, maintenance and personnel.

Keywords: swine farm wastewaters, biological treatment, vermifiltration, hydroponic crops, effluent recirculation.

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List of Abbreviations

ANOVA: analysis of variance

AOB: ammonia-oxidizing bacteria

BOD: biological oxygen demand

BOD_5: 5-day BOD

COD: chemical oxygen demand

DWC: deep-water culture

EC: electrical conductivity

FISH: fluorescent in-situ hydridization

HLR: hydraulic loading rate

HP: hydroponics, or hydroponic

HRT: hydraulic residence time (also known as hydraulic retention time)

LECA: light expanded clay aggregate

LOQ: limit of quantification

NFT: nutrient film technique

NOB: nitrite-oxidizing bacteria

OLR: organic loading rate

RC: relative change

RWW: raw wastewater

SD: standard deviation

SE: standard error

SMEWW: Standard Method(s) for the Examination of Water and Wastewater

TDS: total dissolved solids

TKN: total Kjeldahl nitrogen

TN: total nitrogen

TOC: total organic carbon

TP: total phosphorus

TSS: total suspended solids

VC: vermicompost

VF: vermifilter, or vermifiltration

WVC: woodchips and vermicompost mixture

WWTP: wastewater treatment plant

List of Symbols

Symbols used in hydraulic parameter calculations

F = liquid volumetric flow

V_{HP} = effective liquid volume in the hydroponic unit

$V_{HP,disp}$ = liquid volume displaced by aeration and floating rafts weight in the hydroponic unit

$V_{HP,max}$ = maximum liquid volume in the hydroponic unit

$V_{VF,void}$ = void volume in the vermifilter

Symbols used in physico-chemical water analysis

General calculations:

x_{feed} = parameter value in the feed of a given compartment

x_{eft} = parameter value in the effluent of a given compartment

Total dissolved solids:

m_{cap} = mass of the capsule

m_{evap} = mass of the evaporated capsule with solids

m_{full} = mass of the capsule filled with water sample

Total suspended solids:

m_{ftr} = mass of the filter

m_{sol} = mass of solids retained in the filter

Symbols used in scale-up calculations

$A_{VF,up}$ = upscaled vermifilter surface area

$A_{HP,up}$ = upscaled hydroponic unit horizontal area

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1. Introduction

1.1 Swine farming sector

World swine livestock headcount was reported to reach 978.3 million heads in 2018, and pork meat production was 120.8 million tons in the same year (FAO, 2020, last accessed on August 28th, 2020). In the 28-state European Union, according to Eurostat, swine livestock amounted to approximately 148 million heads. In Portugal, the latest Eurostat data (2016) report 1510 specialist pig farms, 910 of which located in the Center region, followed by Alentejo (280) and Algarve (90). A standard output of €290.7M was reported for this sector in Portugal in 2016. The number of animals has been increasing from 2010 to 2019, reaching 2.2 million heads in 2019. Of those, 239 thousand breeding pigs, 234 thousand breeding sows and circa 801 thousand piglets weighing less than 20 kg were counted (European Commission, 2020, last accessed on August 28th, 2020). According to the Portuguese National Statistics Institute (Instituto Nacional de Estatística - INE), pork meat production reached 383,000 tons in 2018. Pork has been the most consumed type of meat in Portugal in the latest years. By inhabitant, 43.4 kg of pork were consumed in 2017, 44.7 kg in 2018, and 44.3 kg in 2019 (INE, 2020, last accessed on August 28th, 2020).

1.2 Environmental impact of swine farming

Modern livestock production techniques changed from a large number of small scattered mixed production farms to fewer large-scale farms, specialized on a certain type of livestock, including cattle, pigs or poultry. The farms needed to increase their size in order to increase productivity and decrease unitary costs. That caused the concentration of single-type production farms on small areas. Feedstuffs must be supplied to a particular area; under the philosophy of large-scale production, the supplied amounts must largely exceed the exact quantities necessary to feed the animals, and the large local output of generated manures, which are costly to transport, needs to be somehow managed in the same area (Backus et al., 1998).

Piggeries are a source of significant environmental impact. Intensive swine farming is associated with the use of large amounts of water for cleaning the facilities and cooling the animals, which live in crowded confined spaces; this creates a wastewater management problem. A study of 168 Chinese small-scale pig farms showed a production of 216 ton/year of manure, 333 ton/year of urine and 773 ton/year of washing wastewater (median values) (Zhang et al., 2017). Swine manure and the generated wastewater were reported to carry 6.3 to 11.6% total nitrogen (TN) and 3.3. to 8.9% ammonia nitrogen (NH_3-N), 1.7 to 4.4% total phosphorus (TP) and 2.2 to 5.5%

total potassium, per dry weight (Choudhary et al., 1996). In another study, undiluted liquid swine manure was reported to contain, on average, 1237 mg/L of total suspended solids (TSS), 310 mg/L TP, 2344 mg/L of total Kjeldahl nitrogen (TKN), 1858 mg/L of total organic carbon (TOC), a chemical oxygen demand (COD) of 10888 mgO_2/L, and a 5-day biochemical oxygen demand (BOD_5) of 6345 mgO_2/L prior to treatment (Chelme-Ayala et al., 2011). Another study reported 2540 mg/L TN, 3080 mg/L TP and 17080 mg/L TOC in liquid swine manures (Antoneli et al., 2019). Lower values were also reported: 75 mg/L NH_3-N, 44 mg/L organic nitrogen, 84 mg/L phosphorus as P_2O_5, and 66 mg/L K_2O (Brumm et al., 2002). Direct use of animal manures as fertilizers is not the best option, as nutrients in manures were found to be of limited availability for crops (only 20% of N, 40% of P_2O_5 and 29% of K_2O available) (Zublena & Barker, 1992). In addition, swine manures are a source of environment contamination with heavy metals. A study reported 0.13 mg/L copper and 0.97 mg/L zinc in wastewater (Zhang et al., 2017). Swine manures also carry pathogenic micro-organisms; one study of undiluted liquid manure reported 1.2×10^6 colony-forming units (CFU) per 100 mL total coliforms and 1.1×10^5 CFU/100 mL faecal coliforms (Chelme-Ayala et al., 2011). If left untreated, pig manure will easily contaminate surface waters, soils and, by leaching through the soil, also groundwaters, causing a negative environmental impact (ecotoxicity, water eutrophication) and posing public health threats. Besides water and soil contamination, pig farms are also an important source of air pollution. Gaseous emissions from livestock farms include greenhouse gases such as CO_2 from animal metabolism, methane and dinitrogen oxide (nitrous oxide, N_2O), hydrogen sulphide, gaseous ammonia, and volatile organic compounds (VOC), causing environmental toxicity and foul odours, and promoting the greenhouse effect (Sarr et al., 2010; Philippe & Nicks, 2015; Phillips et al., 2016).

1.3 Piggery wastewater treatment

Wastewaters from pig farms can be treated by a variety of methods. The simplest way is direct land application of the slurries. The most common first treatment stage is deposition in anaerobic lagoons for mineralization of organic matter, sometimes after the removal of solids. This is a cheap treatment technique (FSA Environmental, 2000), which understandably makes it the most widely applied. Lagoon treatment is unable to remove and rather increases the amounts of inorganic nitrogen and phosphorus contaminants, generating effluents that have been reported to contain 520 mg/L TSS, 327 to 365 mgTKN/L, 236 to 347 mgNH_3-N/L, a COD of 740 to 869 mgO_2/L, and 82 mg/L TP (Szögi et al., 1997, 2004). In addition, lagoons have a limited holding capacity and, when uncovered, are prone to overflowing under heavy precipitation. The

removed solids can be further treated by composting or vermicomposting (FSA Environmental, 2000), and further wastewater treatment is usually done at wastewater treatment plants (WWTP), either on or off site, requiring transportation. Nitrate removal in WWTP's is achieved through enhancement of denitrification activity by providing a suitable carbon source such as methanol (Timmermans & Van Haute, 1983; Yamashita & Yamamoto-Ikemoto, 2014). Phosphorus is removed by combinations of precipitation, sorption and biological uptake processes (Bunce et al., 2018; Yeoman et al., 1988). Facultative or aerated lagoons are better at promoting oxidation to ensure nitrification and to remove toxic substances and offensive smells, but are much more expensive as they require installation of aerators (FSA Environmental, 2000; Szögi et al., 2004). Several treatments of anaerobic lagoon-held swine wastewater were tested by Szögi and co-workers. A treatment of anaerobic lagoon water by overland flow through an artificial isolated plot of soil with vegetation was reported to allow TN removal efficiencies of 36 to 42%; initial nitrification up to 30% of the initial ammonia content was reported to later decline, possibly due to soil saturation (Szögi et al., 2004). Constructed wetlands have been proposed as a treatment stage to remove inorganic nitrogen and phosphorus and to remove some toxic pollutants such as metals and organic compounds, but like all nature-based systems, they are naturally limited (Abdel-Sabour, 2014; Mora-Orozco et al., 2018). In constructed wetlands, contaminant removal efficiencies depend on the organic load; a detailed study showed removal efficiencies 76 to 86% COD, 72 to 80% TKN, 83 to 90% ammonia, 78 to 91% TP, and 33 to 64% total dissolved solids (TDS) at a 10-h hydraulic residence time (HRT) and initial COD of 400 to 800 mgO_2/L, all but COD removal decreasing at a higher initial COD of 1200 mgO_2/L (Mora-Orozco et al., 2018). Controlled digester tanks can also be used but they are sophisticated and expensive systems (FSA Environmental, 2000). Some reported average parameters of digester effluents after the treatment of swine wastes were 2600 mgO_2/L COD, 145 mg/L TN, 69 mg/L NH_3-N , 70 mg/L TP. Liquid effluents from digesters can be treated by trickling filters filled with an adsorbent material to remove suspended organic matter, which has been reported to be an efficient treatment for digester effluents at input flows higher than 2 L/min. The trickling filters were reported to remove COD with an average efficiency of 93%, 48% TN, 98% ammonia, and 58% TP (Terán et al., 2017). Treatment of anaerobic lagoon-treated pig wastewater by trickling filters that allowed proliferation of nitrifying bacteria, with pH correction to nitrification optima (7.5 to 8.5), showed removal efficiencies of about 69% for TKN and ammonia, 12% for TN, and a nitrification efficiency of 57% (Szögi et al., 2004). Finally, reactors with an active immobilized culture of nitrifying bacteria, previously acclimated to pig wastewater high ammonia conditions, showed ammonia removal and nitrification efficiencies increasing with HRT values up to 94% and 100% for HRT = 24 h (Szögi et al., 2004). Table 1.1 summarizes the

examples presented above. Considering the technical sophistication necessary to overcome multiple limitations, the search for truly efficient and effective livestock slurry and wastewater treatment systems still continues. Some promising biological treatment techniques involve the use of earthworms to treat animal farming wastewaters and slurries, as will be detailed in the next sections.

Table 1.1: Reported treatment efficiencies for swine wastewater and slurry. AnL: anaerobic lagoons; OF: overland flow; CW: constructed wetlands; TF: trickling filters; Dig: digesters; ICR: immobilized cells reactors.

Treatment	Removal efficiency (%)						Reference
	TDS	COD	TN	TKN	NH_3-N	TP	
OF after AnL			36 to 42		30 (nitrification)		(Szögi et al., 2004)
CW after AnL	60 to 72	76 to 86		72 to 80	83 to 90	78 to 91	(Mora-Orozco et al., 2018)
TF after AnL			12	69	69; 57 (nitrification)		(Szögi et al., 2004)
TF after AnL+Dig		93	48		98	58	(Terán et al., 2017)
ICR after AnL					94 to 100		(Szögi et al., 2004)

1.4 Earthworms in waste treatment

The use of earthworms in the management of different kinds of residues and wastewaters has been widely reported for decades. They have been reported to find use in processing animal wastes, domestic waste, primary sewage, vegetable wastes from food industry, textile fibers, and paper mill pulp and sludge (Edwards & Bater, 1992; Elvira et al., 1997; Elvira et al., 1996a, 1996b; P. Garg et al., 2006; Gupta & Garg, 2008). Vermicomposting of different types of animal manures (V. K. Garg et al., 2005, 2006) and, specifically, vermicomposting of pig manure (Aira & Domínguez, 2008; Gómez-Brandón et al., 2011) and worm-assisted treatment of wastewater from swine facilities (Li et al., 2008; Manyuchi et al., 2019) have also been studied. The possibility to process wastes in this manner into suitable organic fertilizer for sustainable agriculture and to process earthworm biomass into highly nutritious feed for animal farming such as pig, poultry and fish have been studied (Edwards & Bater, 1992). Tested earthworm species include *Lumbricus terrestris* L. (Hanna & Weaver, 2002), *Eudrilus eugeniae* (Kinberg), *Perionyx excavatus* (Michaelsen), *Dendrobaena veneta* (Rose) (Edwards & Bater, 1992) and, most commonly, *Eisenia* species such as *E. fetida* (Sav.) (Edwards & Bater, 1992; Elvira et al., 1996a; 1996b).

1.4.1 Earthworm biology

Earthworms belong to the Lumbricidae family of the order Opisthophora, class Clitellata (also known as Oligochaeta), phylum Annelida (ITIS, 2020, last accessed in October 2020). Annelids (Figure 1.1) are characterized by an elongated body, composed of multiple segments that are

separated by septa. On the outside the body is protected against desiccation by a thin cuticle aided by slime secretion. They have a relatively sophisticated digestive system, with a mouth, a muscular pharynx, an oesophagus, a crop, a gizzard, an intestine and an anus. The circulatory system consists of a dorsal vessel, conducting the blood in the forward direction, and a ventral vessel, where the blood flows backwards; both are connected by a set of muscular "hearts" that propel blood from the dorsal into the ventral vessel; the circulatory system also has smaller ramifications and even capillaries. Annelids also have well developed muscular, excretory, nervous and reproductive systems (Villee & Dethier, 1971).

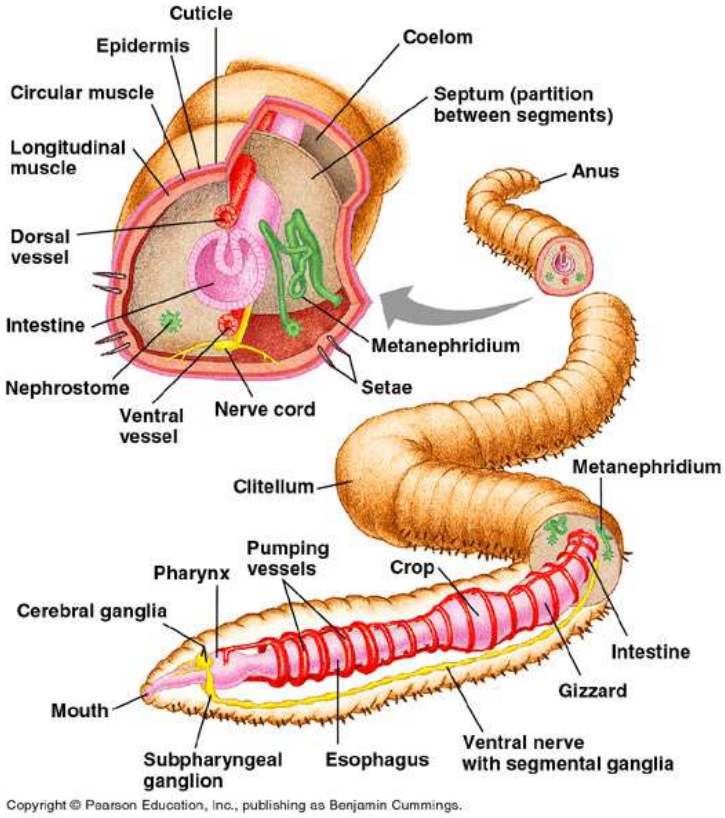

Figure 1.1: Annelid (earthworm) external and internal anatomy (Campbell & Reece, 2005).

From the reproductive point of view, Lumbricids are classified as cross-fertilizing hermaphrodites, meaning that reproduction involves mating of two individuals that reciprocally fertilize one another (Díaz Cosín et al., 2011), although uniparental reproduction involving self-fertilization has also been described (Domínguez et al., 2003). Earthworms have sets of both male and female reproductive organs, opening to the outside in different groups of body segments. Ordered from front to back, the openings include spermatheca pores, ovary pores and male genital pores (Díaz Cosín et al., 2011; Grove & Cowley, 1926; Villee & Dethier, 1971). When the worms mature sexually, they develop some external organs involved in fertilization,

such as the clitellum, tubercula pubertatis and sexual papillae with special genital chaetae and chaetal glands. Copulation occurs with both worms assuming a typical head to tail position (Grove & Cowley, 1926; Villee & Dethier, 1971). Tubercula and genital chaetae secure the pair's copulative union. In most Lumbricids, during coition seminal groves conduct the semen from male pores of one individual to the spermatheca (receptacles for the partner's semen) pores of the other (Díaz Cosín et al., 2011). After sperm transfer, the clitellum produces a slime tube; the worms separate and each worm moves out of the slime tube backwards, making it first pass over the ovarian pores to collect the worm's own eggs and then over the spermatheca pores to collect the partner's semen; when the slime tube slides off, it forms a cocoon that will enclose the reproductive cells and developing embryos (Villee & Dethier, 1971).

1.4.2 Earthworm ecological classification

Earthworms can be classified in groups based on their ecological characteristics: distribution in the soil, preferred physical conditions, feeding habits and adaptations through morphological structures. According to Bouché, from this point of view they are classified into epigeic, anecic and endogeic groups (Bouché, 1977).

Endogeic earthworms

Endogeic earthworm species lack pigmentation, can have variable sizes, present weakly developed lubrication, variable somatic regeneration, quiescence in less favourable seasons, display modest reproduction and maturation rates, and are highly lucifugous. They live in mineral soil layers and feed on mixed mineral-organic soil, thus avoiding coming to the surface and protecting themselves both from weather and surface predators (Bouché, 1977).

Anecic earthworms

Earthworms of the anecic group have darker colour and can reach much larger sizes than epigeic worms (up to 1.1 m), produce abundant mucus for external lubrication, have a good regenerative capacity and relatively slow reproduction and maturation, also present slow mobility; they pass unfavourable seasons in true diapause (dormancy). Theses worms feed on soil surface organic matter but explore soils in depth, digging galleries as deep as 6 meters; the large body size allows the worms to stay partly buried while feeding and quickly withdraw the body in case of predator attack (Bouché, 1977).

Epigeic earthworms

Epigeic group earthworms are characterized by a homochromic pigmentation (adapted to the predominant colour of the milieu), relatively small size, no regenerative capacity, well developed external lubrication by mucus, encystment in cocoons, fast reproduction and quick maturation,

high mobility. Epigeic earthworms frequently live in soil layers rich in organic matter, typically under and within the forest litter of decaying leaves, bark and other decomposing vegetable matter, thus living directly inside their food; on the other hand, not tending to hide deeper in the soil, they are subject to threats from climate and predators, and their colour, size and reproductive strategy are adapted to these threats (Bouché, 1977).

Compost earthworms

Compost earthworms are a fourth group sometimes distinguished from epigeic earthworms for living almost exclusively in compost heaps of decaying vegetable matter like leaves and domestic waste (Sims & Gerard, 1999). Members of this group present a particular interest for the present work.

Eisenia fetida

The genus *Eisenia* is among the typical representatives of the epigeic group (Bouché, 1977) or, according to other sources, compost group (Sims & Gerard, 1999). *Eisenia fetida* (Savigny) is a well-known member of this genus. It is native to Europe but has been introduced in other continents. It is a rather widespread species, found in garden compost heaps, manure-fertilized grassland and sewage filter beds (Sheppard et al., 2014).

Eisenia fetida has shown to be well suitable for organic waste management for its ubiquity, temperature and moisture tolerance, and tendency to dominate in mixed populations (Edwards & Bater, 1992). In a study conducted by Venter and Reinecke (Venter & Reinecke, 1988) with *Eisenia fetida* living on cattle manure, the worm's lifecycle from fertilization to sexual maturity and first cocoon production was reported to last for about 60 to 80 days overall. After a 23-day incubation period, hatchlings emerged from the cocoons. Growth was slow during the first 60 days after hatching, accelerated till day 90 and then slowed down again. All worms were reported to develop a mature clitellum between 60 and 80 days from the start of the experiment. After that, the fertile period was observed to last for more than 500 days. These observations were made under controlled conditions: sifted manure, free of urine, temperature kept at 25°C, humidity between 70% and 80% (Venter & Reinecke, 1988). This was consistent with the reported optimal conditions for this species: temperature between 20 and 29°C, pH close to neutral, moisture from 70 to 85% and salinity lower than 0.5% by mass for most soluble salts (Kaplan et al., 1980).

1.4.3 Vermicomposting

A simpler way to process residues using earthworms is the so-called vermicomposting, where worms live in moist solid residues and process them by feeding and excreting products of their metabolism (vermicast). At laboratory scale this involves inoculating residue-filled containers with live earthworms and letting the treatment progress under appropriate temperature and moisture conditions, and in the dark. The initial adaptation phase may be conducted on a smaller scale, and then the acclimated individuals can be transferred into larger vessels (Elvira et al., 1996a, 1996b; Gupta & Garg, 2008; Yadav et al., 2010). This way, vermicomposters function as simple batch type bioreactors.

A large number of studies on vermicomposting as a technique to treat wastes have been performed. Vermicomposters can be constructed in a simple way as containers filled directly with the residues to be treated (Elvira et al., 1997; Elvira et al., 1996a, 1996b; V. K. Garg et al., 2006), or they can contain layers of inert substrate, such as gravels and sand underneath the earthworm inhabited layers containing organic matter (Jain et al., 2003; Villar et al., 2016). The residues for treatment may be mixed with soil (P. Garg et al., 2006; Jain et al., 2003; Yadav et al., 2010) or vermicompost (Aira & Domínguez, 2008; Hénault-Ethier et al., 2016; Pereira et al., 2014; Yadav et al., 2010) to help the worms' acclimation and activity. Vermicomposters are usually kept at temperatures from 15 to 25°C, moisture ranging from 55 to 85%, and in the dark to create the most natural possible conditions for the worms (Elvira et al., 1996a, 1996b; P. Garg et al., 2006; V. K. Garg et al., 2006).

1.4.4 Vermifiltration

A technological upgrade to vermicomposting techniques consists in turning vermicomposting reactors into trickling filters filled with solid matter and inhabited by earthworms and micro-organisms. This way they function as trickle-bed bioreactors, through which liquid wastes pass at a flow rate that will allow for their biotreatment under continuous operation, as opposed to the discontinuous treatment by vermicomposting.

Vermifilters are constructed similarly to vermicomposting reactors. Usually they are opaque containers filled, from bottom to top, with gravels of decreasing size (Sinha et al., 2007, 2008, 2012; Wang et al., 2013), sand (Lourenço & Nunes, 2017b, 2017a; Wang et al., 2013) and a worm bed, which can contain soil (Sinha et al., 2007, 2008, 2012), vermicompost (Li et al., 2008; Lourenço & Nunes, 2017b, 2017a; Sinha et al., 2012), woodchips (Li et al., 2008) and/or sawdust

(Lourenço & Nunes, 2017a). Wastewater is fed on top of the vermifilters at a flow that dictates the hydraulic loading rate (HLR), the organic loading rate (OLR) and the hydraulic residence time (HRT) in the filter. These parameters need to be set to keep the necessary moisture content and prevent flooding; they have been shown to influence wastewater treatment efficiency (T. Kumar et al., 2014; Lourenço & Nunes, 2017b). Vermifiltration systems allow for further improvements such as effluent recirculation (Li et al., 2008; Lourenço & Nunes, 2017b) and the use of several consecutively connected vermifilters (Lourenço & Nunes, 2017b).

1.4.5 Effects of waste treatment by earthworms

Vermicomposting and vermifiltration have been studied as tools to treat domestic waste (e.g. P. Garg et al., 2006; Jain et al., 2003; Sinha et al., 2008), dairy wastewater (Sinha et al., 2007), animal manure and wastewaters (Aira & Domínguez, 2008, 2009; Elvira et al., 1996a; Li et al., 2008; Mitchell, 1997), and effluents from industry (e.g. Elvira et al., 1997; Elvira et al., 1996b; P. Garg et al., 2006), including petroleum contaminated waters (Sinha et al., 2012). In recent detailed reviews on the matter, several aspects of earthworm action were identified: mechanical action of comminution of particles to smaller size and mixing through burrowing and grinding of substrate and production of vermicast; physical effects of increased surface area, increased filtration efficiency and vermicast sorption properties; chemical and biochemical effects of gut digestion and vermicast biologically active substances; microbial inoculation of the substrate with vermicast bacteria (Samal et al., 2017b; Singh et al., 2017). The most studied earthworms (e.g. *E. fetida*, *E. andrei* and *Lumbricus terrestris*), together with associated micro-organisms, have been reported to efficiently degrade organic matter to inorganic products, nitrify ammonia and destroy faecal micro-organisms. Earthworms excrete mucus that contains digestive enzymes with organic compounds decomposing activity (Singh et al., 2017). Waste-decomposing earthworms present high amylase (Bamidele et al., 2014; Prabha et al., 2007), cellobiase, endoglucanase, phosphatase, nitrate reductase (Prabha et al., 2007) and lipase (Bamidele et al., 2014) activities in their digestive system. Earthworm mucus also presents several cellulose-degrading enzyme activities (Lattaud et al., 1999). However, in the case of *E. fetida*, there is evidence that fungi present in the worm's gut and vermicompost play a role in cellulolytic activity rather than the worm itself (Aira et al., 2006). According to multiple studies, organic matter is removed from different types of waste and wastewater with efficiencies up to 58 to 63% TOC (Gupta & Garg, 2008; Yadav et al., 2010), 98 to 99% BOD_5, and 70 to 92% COD (Lourenço & Nunes, 2017a, 2017b; Manyuchi et al., 2013; Sinha et al., 2007). Some of the organic matter is reported to be converted to humic substances (Elvira et al., 1996b; Pereira et al., 2014;

Yang et al., 2014). Earthworm treatments have also been shown to remove ammonia up to 67% by vermicomposting and up to 59% by vermifiltration (Rajpal et al., 2014), and increase TKN in different wastes from 30% to 5.8-fold by vermicomposting (P. Garg et al., 2006; V. K. Garg et al., 2006; Gupta & Garg, 2008). Nitrate increase was reported to be up to 75% by vermicomposting and 187% by vermifiltration (Rajpal et al., 2014). Total phosphorus increased up to 201% (V. K. Garg et al., 2006; Rajpal et al., 2014; Suthar, 2009), and, according to another study, up to 6.5-fold (P. Garg et al., 2006). Widely variable efficiencies were reported for different waste types and compositions and different treatment technologies. Due to the loss of carbon as CO_2, the C:N ratio has been reported to decrease in solid and liquid wastes treated by earthworms (Elvira et al., 1996b; V. K. Garg et al., 2006; Gupta & Garg, 2008; Suthar, 2010). Mineralization of organic matter can result in increased electrical conductivity (EC) (Gupta & Garg, 2008), although in some studies a decreased EC was recorded (V. K. Garg et al., 2006; Yang et al., 2014). In vermifiltration, a series of layers of solid materials retains pollutants both by mechanical filtration and by adsorption, thus helping their conversion by earthworms and micro-organisms. The general mechanism of wastewater pollutants removal by vermifiltration, including retention by the substrate layers, solids processing and digestion by the worms, and bacterial metabolic action, was summarized by Singh and colleagues as represented in Figure 1.2 (Singh et al., 2017).

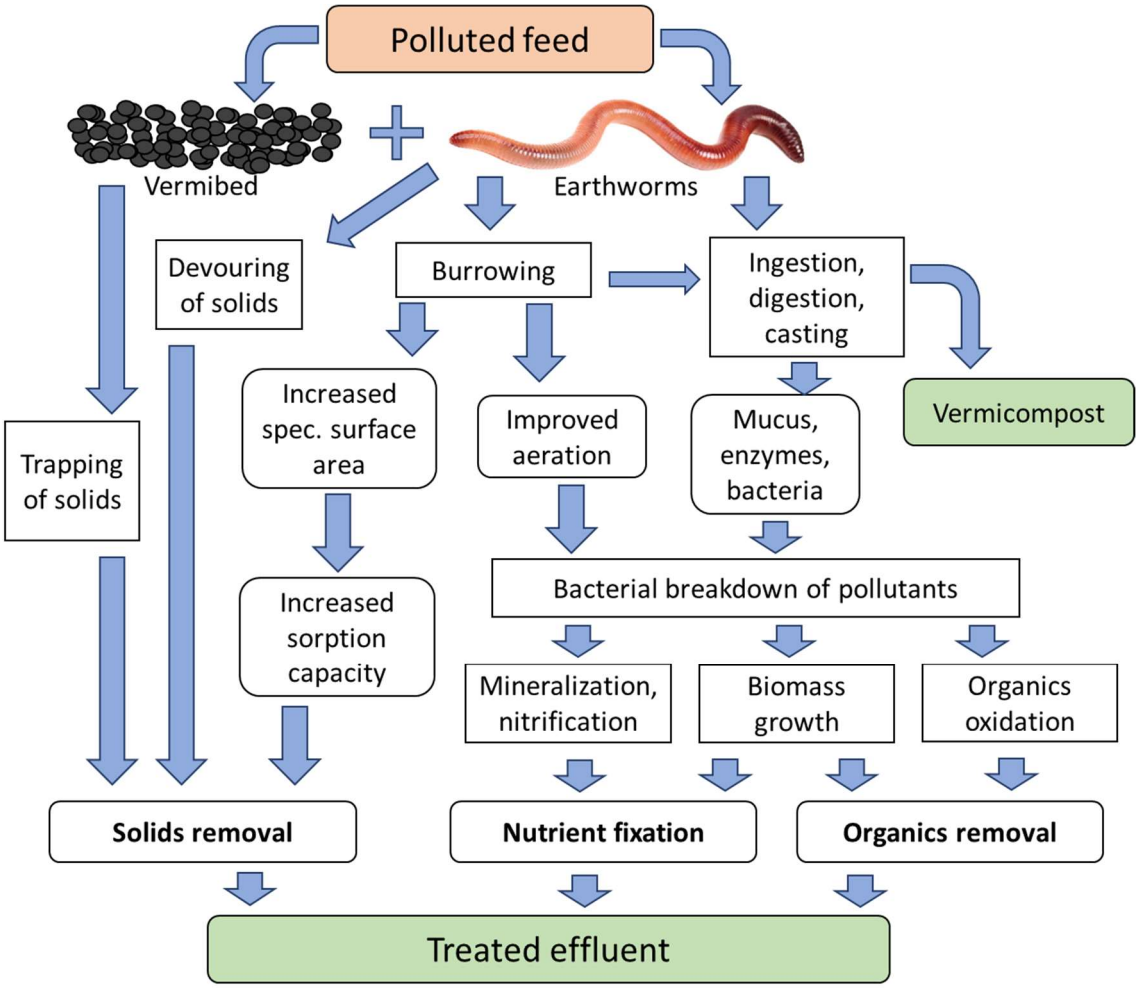

Figure 1.2: Schematic representation of different mechanisms of wastewater treatment by vermifiltration (Singh et al., 2017).

Finally, earthworm treatment alters microbial communities, favouring betaproteobacteria (Castillo et al., 2013; Huang et al., 2017; Wang et al., 2013), a class that includes ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and some of the denitrifiers (Nielsen et al., 2009); on the other hand, other micro-organisms such as fungi (Pereira et al., 2014; Villar et al., 2016) and gammaproteobacteria (Castillo et al., 2013), including human pathogens *Salmonella* and coliforms (Flack & Hartenstein, 1984; Yadav et al., 2010) are suppressed.

Presence and activity of *Eisenia fetida* was shown to have suppressing effects on pathogenic bacteria *Bacillus sp.*, *E. coli*, *Serratia marcescens* (Edwards & Fletcher, 1988), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* and fungi such as *Candida albicans*, *Aspergillus flavus* and *Aspergillus niger* (Andleeb et al., 2016). Part of the effects on the micro-organisms are due to intrinsic earthworm action (Edwards & Fletcher, 1988; Flack & Hartenstein, 1984; Sinha et al., 2014). Intrinsic earthworm antimicrobial action in

Eisenia andrei, for instance, involves entrapment in the mucus and the consequent starvation, and also digestion by enzymes both within the gut and in the vermicast (Singh et al., 2017). Increased activities of worm enzymes degrading microbial cell walls and proteins were reported in response to microbial challenge (Procházková et al., 2013). Besides the earthworm physiology itself, the effects on the microbiome are also due to bacteria inhabiting vermicompost, as observed in vermicompost reactors without worms (Hénault-Ethier et al., 2016; Singh et al., 2017). Actinobacterial populations have been reported to increase during the treatment vermicomposting of vegetable wastes (Domínguez et al., 2019). Actinobacteria are known to produce a great diversity of natural antibiotics, and also fungicides, herbicides and anthelmintic agents (Barka et al., 2016).

The diversity of mechanisms by which earthworms can destroy micro-organisms during waste and wastewater treatment were summarized by Singh and co-workers (Figure 1.3) (Singh et al., 2017).

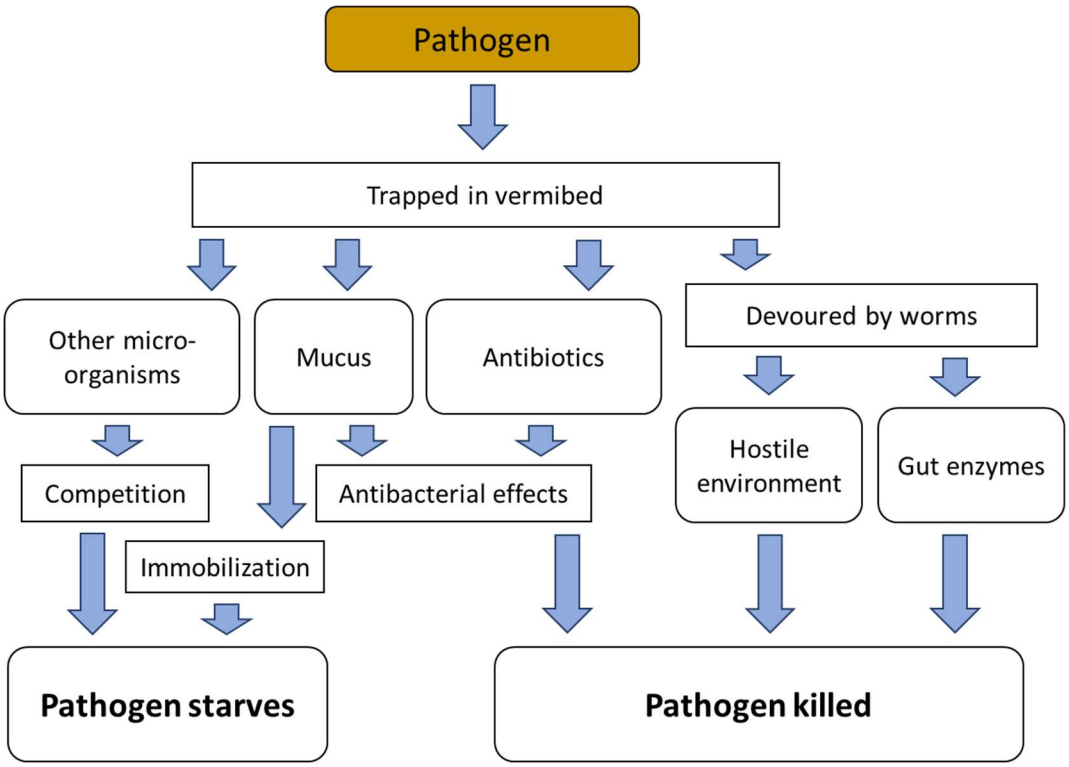

Figure 1.3: Diagram representing mechanisms of pathogenic micro-organism removal in vermifilters (Singh et al., 2017).

A number of studies have been performed to test the interaction of earthworm waste treatment with plant growth. In one study *Canna indica* plants grown directly on vermicomposting reactors surface were reported to significantly improve the removal of TN and NH_3-N, and also somewhat

aid the removal of BOD, COD and solids; effluent phosphate did not change significantly due to the presence of plants (Samal et al., 2017a). On the other hand, products of earthworm treatment have been reported to be beneficial to plants. In a study where hydroponic nutritional medium was amended with humic acids, the authors reported improvements in photosynthetic rate and mesophyll conductance due to humic acid presence (Haghighi & Teixeira Da Silva, 2013). Humic acids are known to be generated in earthworm waste treatments (Elvira et al., 1996b; Pereira et al., 2014; Yang et al., 2014). In a work investigating the effects of earthworm-treated wastewater on the germination of *Allium cepa* (onion) roots, an accelerated germination was reported on worms-treated wastewater while untreated wastewater retarded it (C. Kumar & Ghosh, 2019). These and numerous other studies (Samal et al., 2017b) suggest the potential of combining earthworm-based waste and wastewater treatment systems with crop cultivation, from both the waste treatment and crop production points of view.

1.5 Soil-less crop production

Soil-less culture, or nutriculture, is a collective term for plant growth techniques in nutrient media other than soil. Water-culture, aggregate culture and adsorbent nutrient techniques are referred to among nutriculture techniques. Hydroponic (water) cultivation consists in growing plants with roots immersed in a nutritive solution. In aggregate culture, plant roots are supported by a solid inert aggregate such as sand, gravel, cinders or vermiculite, and the nutritional solution flows in between the inert particles. Adsorbent nutrient technique differs from both others by providing nutrients adsorbed on a solid ion-exchange material, only needing the addition of water (Hoagland & Arnon, 1950).

Nutriculture is at least as old as 1699, and modern water-culture technique dates back to 1860, when it was used in plant physiology studies by J. von Sachs (Hoagland & Arnon, 1950; Sachs, 1887). It has since been widely used as a research technique. Hoagland and Arnon published a circular on water-culture methods at the California Agricultural Experiment Station in 1938, revised in 1950. They pointed out the possibility of growing high value crops in places with poor soil, a better control of plant disease agents through the use of synthetic media, and the relatively easy installation without depending on land (for example, in military camp conditions) as the most important advantages of such techniques. The authors cautioned about some drawbacks, stating that nutriculture could get costly, had sometimes low yields, needed expert knowledge unfamiliar to traditional farmers, did not guarantee sanitation or water saving, and did not improve nutritional value of produce (Hoagland & Arnon, 1950). Later studies, focused on economic analysis, have reported better water use efficiency than in conventional farming

(Barbosa et al., 2015), but pointed out high installation costs and high energy consumption to be the major factors compromising the sustainability of soil-less cultivation (Barbosa et al., 2015; Uddin & Dhar, 2018). Hoagland and Arnon's circular provided important technical guidance on nutriculture implementation, including recipes for nutrient solutions (now called Hoagland solutions) for normal use and for nutrient deficiency demonstration in scientific research (Hoagland & Arnon, 1950).

1.5.1 Soil-less cultivation techniques

Soil-less cultivation can be divided into two main technologies: open systems, where water and nutrients must be regularly supplied to the crops and a large amount of effluent is generated, and close-loop systems, where most water is recirculated and thus allows to substantially improve the water use footprint (Putra & Yuliando, 2015; Sommerville et al., 2014). Regarding the crop contact with the nutritional medium, several system configurations have been developed.

Wick systems are among the simplest hydroponic cultivation techniques. In wick systems, plant pots are placed in an elevated position above the nutrient-rich water level. Nutrient solution is conducted to plant roots by capillarity through wicks immersed in the water below (El-Kazzaz, 2017).

Soil-less cultivation on solid substrates beds, or medium bed, involves inert substrates that fill up the whole planting compartment volume. These substrates provide support to roots (El-Kazzaz, 2017; Sommerville et al., 2014) and provide a large surface for the development of beneficial bacteria (Sommerville et al., 2014). The support material can be mineral, like volcanic gravel, expanded clay, perlite, vermiculite, or rockwool, or organic, such as tree barks, sawdust, fleece, or coconut fiber (El-Kazzaz, 2017).

Deep-water cultivation (DWC), raft or floating bed systems, are a type of hydroponic system where plants are placed in holes in floating platforms on the nutrient-containing water surface. This sort of systems are suitable for leafy plants, herbs and strawberries (El-Kazzaz, 2017; Sommerville et al., 2014).

Drip systems comprise a top container with plants on inert substrates and a bottom container with nutrient solution, aerated by air pumps or compressors. A water pump delivers water with nutrients from the bottom container to plants through drippers, allowing it to percolate down to the roots and then back into the water reservoir. This is suitable for plants with large root balls (El-Kazzaz, 2017).

In nutrient film technique (NFT), plants are usually grown in channels where a thin film of oxygenated nutrient solution flows continuously, being pumped from an aerated reservoir. The channels are placed with a slope that allows the adequate drainage. This technique is effective for herbs, strawberries and leafy plants with small root systems (El-Kazzaz, 2017; Sommerville et al., 2014).

Ebb and flow systems are more sophisticated, providing an alternate regime of flooding and draining of the plant compartments, located above the water reservoir. Water with nutrients is pumped from the reservoir, and an overflow pipe with a siphon drains the upper compartment once a certain level is reached. This is done to allow the roots to alternately receive water-dissolved nutrients and oxygen from the atmosphere. The water pump can be automatically turned on and off to set the flooding-draining rhythm (El-Kazzaz, 2017; Sommerville et al., 2014). This sort of water and air supply regime is also used in medium bed systems, filling and emptying the spaces between pieces of substrate (Sommerville et al., 2014).

Finally, aeroponic systems are those where plants hang above the nutrient-rich water container through an opaque perforated platform, and their roots are irrigated by a fine water mist, sprayed around them at regular times through a network of tubes with sprayer heads. Pressure is created either by a water pump or an air compressor, in this case requiring the water compartment to be airtight (El-Kazzaz, 2017).

1.5.2 Water quality requirements

Since in soil-less cultivation systems the roots get their nutrients directly from the contacting water, its quality and composition are critical for successful growth. Most plants show a pH tolerance range between 5.5 and 7.5; outside this range, plants may experience decreased nutrient availability and growth impairment (Putra & Yuliando, 2015; Sommerville et al., 2014). For instance, potassium, phosphorus and sulphur are poorly absorbed at lower pH; iron and manganese at higher pH; and other elements such as nitrogen, calcium, magnesium and, less severely, copper and zinc, become less available to plant roots both at high and low pH (Sommerville et al., 2014). Electric conductivity (EC) is also an important parameter, generally acceptable between 1.5 and 2.5 mS/cm (0.15 to 0.25 S/m) (Kumari et al., 2018; Putra & Yuliando, 2015). Oxygen dissolved in water contacting the roots is essential for their respiration and needs to be kept at 3 mg/L minimum. Temperature depends on each plant's seasonal adaptation, winter crops typically requiring lower temperatures. Leafy green vegetables grow best at 14 to 20°C, tending to bolt, flower and seed at higher temperatures. Water temperature is more important than air temperature in the facilities (Sommerville et al., 2014).

1.5.3 Using hydroponics in wastewater treatment

Studies of hydroponic plant cultivation as a natural wastewater treatment method have been published, and the findings have varied greatly for different plant species under different conditions. A study of hydroponically grown lettuce using domestic greywater, pre-treated by biofiltration within the same facility, showed the viability of small-scale greywater treatment installations coupled with hydroponic growth (Eregno et al., 2017). In another study, phosphorus-supplemented pig wastewater used as nutrient solution for gravel bed hydroponic growth of the giant reed *Arundo donax* was reported to result in a faster growth than in soil and a stem accumulation of nitrogen increased by 3%, magnesium by 28%, copper by 25% and zinc by 29%; however, phosphorus, potassium, calcium, and iron accumulation was lower than in the soil culture system (Mavrogianopoulos et al., 2002). In a study of fish aquaculture wastewater treatment in a combined aquaculture-hydroponic (aquaponic) facility, hydroponically growing lettuce was efficient in reducing TDS, COD, TKN, phosphorus and potassium, while pH was not significantly affected, and dissolved oxygen increased. Treatment efficiency increased with increasing HRT (Keeratiurai, 2013). Hydroponic growth of the wetland plant *Typha latifolia* (common cattail) on brewery wastewater was reported to result in good plant growth and removal of 54 to 80% TKN, 42 to 65% ammonia, 47 to 58% nitrate and 51 to 70% phosphate (Gebeyehu et al., 2018). In a study of an experimental municipal wastewater treatment plant in Poland with a hydroponic facility growing water plants for tertiary treatment, the introduction of a hydroponic stage as a tertiary treatment caused no difference in the removal of TN and NH_3-N, TP and phosphate, but decreased final nitrate by 11% and nitrite by 46% relatively to the system without the hydroponic stage (Bawiec, 2019). A pilot-scale wastewater treatment plant coupled with hydroponic cultivation of several plants such as squash, beans, sweet corn, eggplants, Cherry tomatoes, rosemary, citrus trees and olives, was reported to remove, depending on the plant species: up to 50% BOD, 45 to 71% COD, up to 47% TN and up to 51% TP (Haddad & Mizyed, 2011). A review by Samal et al. (2007b) referred to the contribution of plants on wastewater micro-organism content and particularly pathogen removal by processes such as mechanical filtration, sorption, sunlight ultraviolet radiation exposure of immobilized pathogens, competition by plants' microbial symbionts, and antimicrobial activity of exudates from roots (Samal et al., 2017b).

These studies pointed out the potential of combining wastewater treatment with hydroponic cultivation of selected crops as a promising nature-based technology to improve water quality and produce food. Thus, the viability of cultivating edible or otherwise commercially relevant

plants hydroponically for wastewater treatment should be thoroughly explored, as well as the health safety of the resulting end products.

1.6 Motivation and main goals

The widespread pork consumption, the importance of the pig farming sector for the Portuguese economy and its associated pollution create a complex problem that needs to be addressed. Portuguese Central region and particularly Leiria district (LUSA, 2017, 2019) have the largest numbers of intensive swine farms (European Commission, 2020). To mitigate the resulting environmental impacts, new sustainable solutions are needed.

The main goal of the present work was to implement a laboratory-scale biological treatment system to study the treatment of pig farm wastewaters held in anaerobic/facultative lagoons. The system was conceived to combine a soil filter containing live earthworms (vermifilter) to remove organic matter, ammonia and pathogens, and a hydroponic plant growth unit to further remove nitrogen and phosphorus by plant assimilation. This work also aimed to simultaneously test edible crops for their ability to grow hydroponically on such vermifiltered wastewaters.

With the aim of recycling as much water as possible during the wastewater treatment process, the design involved the use of most of the final effluent to dilute the raw wastewater in the feed.

Finally, this work also aimed to theoretically reflect on the possibility to upscale the system for use at a real pig farm, based on the treatment results.

1.7 Thesis outline

The main text of this thesis comprises the following chapters:

- **Introduction**;
- **Material and Methods**, where the experimental setup and analytical procedures are described;
- **Results and Discussion**, where the experimental results are presented and analysed critically based on the available scientific information;
- **Proposal of a Real-Scale Treatment System for Piggeries**, describing a proposed construction and sizing of a real-scale treatment system based on the pilot-scale system used in this work;

- **Conclusions**, where the most relevant conclusions from the presented results are summarized;
- **Future Work**, which suggests the work needed to answer the questions and address the problems brought up by the results presented above.

In addition to the main text, there are two **Appendices**, containing information that was not considered essential for the interpretation of results but can complement the data presented in the main text.

In the **Results and Discussion** chapter of this thesis, the conception and construction of a laboratory-scale system sequentially combining a vermifilter and a hydroponic growth unit is presented. The operation and monitoring of the implemented system was divided in four experimental periods.

During the experimental period 1, the results of the physico-chemical analyses (solids, electrical conductivity, pH, COD, BOD_5, total nitrogen, ammonia, nitrite, nitrate and phosphorus) of swine farm wastewater treated by vermifiltration are presented and discussed, in order to confirm the data available in literature.

During the experimental period 2, described and discussed in the next section, crops were selected and introduced in the hydroponic unit.

The following sections refer to the experimental periods 3 and 4. During experimental period 3, the hydroponic unit was fully occupied with the selected crop while still using the same vermifilter and recirculating water from periods 1 and 2. During period 4, the system had been fully cleaned and restarted with vermifiltration and hydroponic growth simultaneously, for comparison with period 3. The introduction of the selected crops in the hydroponic unit, and the monitoring of plant growth, health and survival are presented. Coliform quantification and AOB and NOB analysis by fluorescent in-situ hybridization (FISH) are also discussed. Lastly, water physico-chemical analysis of electrical conductivity, pH, BOD_5, ammonia, nitrite, nitrate, total nitrogen and phosphorus are presented.

In the next sections, the limitations and problems encountered throughout the experimental work are discussed.

In the chapter **Real-Scale Treatment Systems for Swine Farms** a theoretical bioreactor approach to the constructed system elements is attempted, and a reflection on the real-scale applicability of a similar system is made.

2. Material and Methods

2.1 System setup

The experimental system built for this study (Figure 2.1, Figure 2.2) comprised the following modules:

- A raw wastewater reservoir;
- A wastewater mixing tank;
- A trap for the retention of larger solids;
- A vermifilter;
- A hydroponic DWC unit;
- A final reservoir for treated water.

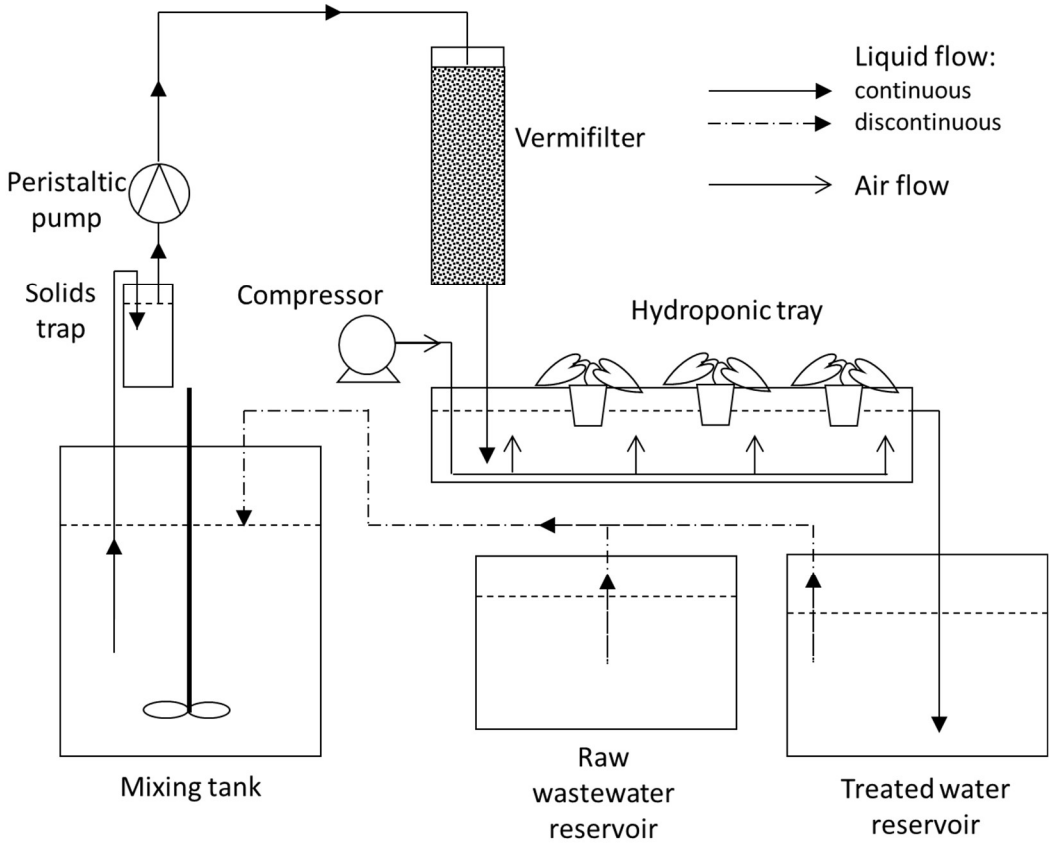

Figure 2.1: Schematic representation of the built vermifiltration-hydroponic system. Arrows indicate the liquid and air flow directions.

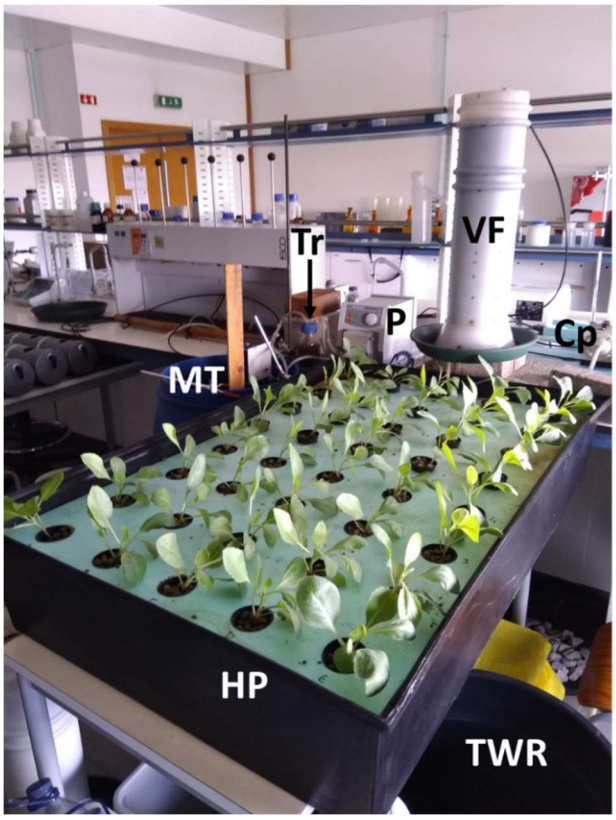

Figure 2.2: The complete system. Cp: air compressor; HP: hydroponic tray; MT: mixing tank; P: peristaltic pump; Tr: trap for solids; TWR: treated water reservoir; VF: vermifilter.

The mixing tank was a cylindrical 160 litre barrel containing a total liquid volume of 100 L, stirred by a 120 W CAT R50 overhead stirrer with a flat-blade impeller (diameter 100 mm, blade width 50 mm) at ~120 rpm. From there, diluted wastewater (see section 2.3) was pumped by a Heidolph Pumpdrive 5101 peristaltic pump calibrated to an estimated average flow of 11 L/day. The wastewater was fed to the vermifilter continuously through a 4-mm plastic tube, dripping onto the top substrate layer.

The vermifilter (Figure 2.3) consisted of an opaque plastic cylinder (total height 63 cm, internal diameter 16 cm) with 4-mm lateral perforations for aeration in the lower half, 6 cm apart, and filled bottom to top with layers of:

- Gravel #4 (22.4 by 45 mm), 10 cm;
- Gravel #2 (16 by 22.4 mm), 7.5 cm;
- Gravel #1 (6.3 by 14 mm), 7.5 cm;
- Gravel #0.5 (2.0 by 6.3 mm), 5 cm;
- Coarse river sand, 3 cm;
- Fine river sand, 10 cm;
- Woodchips-vermicompost mixture (WVC) with one measure (dry apparent volume) of vermicompost (Earthworm humus, Siro^{TM}) by two measures of wood chips obtained

from a local sawmill, 15 cm. This layer was inoculated with live *Eisenia fetida* earthworms as described in section 2.2.

Water flowed through the vermifilter by gravity, being collected at the bottom in a circular tray, from where it trickled, through an 8-mm perforation, into the hydroponic unit placed below. It was ensured that the flow through the vermifilter was free enough to be limited by the peristaltic pump alone.

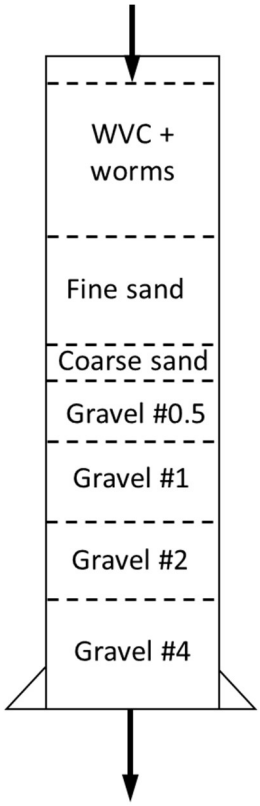

Figure 2.3: Schematic representation of the vermifilter layers. Wastewater entry and exit points are represented by the arrows.

The deep-water culture (DWC) hydroponic unit (Figure 2.4) comprised a rectangular 120 cm long, 80 cm wide, 25 cm high black plastic tray, open at the top. Water inside was aerated by a mesh of perforated 4 mm plastic tubes, forming 20-cm side squares, fed by a 60 W, 70 L/min Hailea ACO-328 air compressor. Water flowed out through a standpipe of defined height, which allowed to set the total volume and hydraulic residence time for a constant volumetric flow.

For crop growth, two polystyrene foam plates were placed on top of the container as rafts floating directly on water surface. Crops were planted in plastic net pots (diameter 55 mm, Bulsø Plastics, Denmark) filled with 7.9(±0.8) mm light expanded clay aggregate (LECA) for root support. The pots were placed in holes of appropriate diameter made in the polystyrene, 13 cm apart.

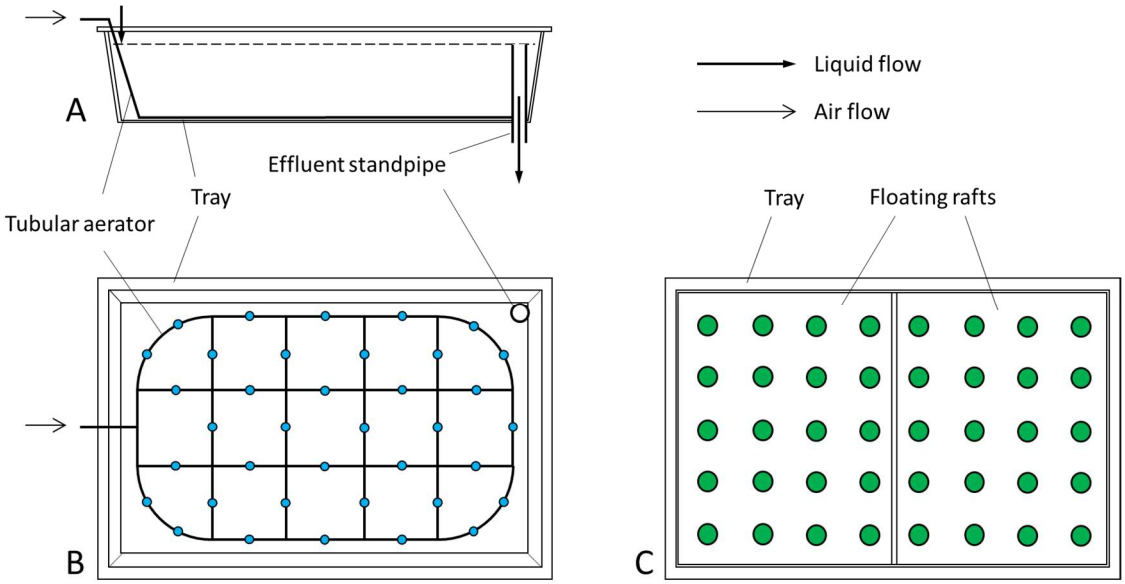

Figure 2.4: Detail of the hydroponic growth unit. A: side view; B: top view of uncovered tray; C: top view of tray with rafts. Arrows indicate water entry and exit points; small blue circles indicate aeration orifices; green circles indicate plant pots placement.

Water exiting the hydroponic growth module flowed into a 75 L reservoir, from where it was returned to the mixing container to dilute the raw wastewater. The dilution was performed in a discontinuous way every seven days, by transferring water from the end reservoir to the mixing tank and adding raw piggery wastewater to achieve the desired dilution (see section 2.3). The rest of the volume in the end reservoir was considered the final treated water and was removed from the system. This 10% dilution was chosen based on

2.2 Earthworms and crops

The first *E. fetida* earthworms were provided by a private earthworm breeder. The worms were placed in a houseplant pot filled up to two thirds with garden soil and topped with vegetable wastes such as banana peels, apple and pear cores, and cabbage leaves. The pot was placed in a liquid-collecting tray and covered loosely with a black plastic bag to allow air in and ensure darkness and moisture. The wastes were renovated every week or two as they decayed and were consumed. The worms had been breeding this way for several months before being used in this study. At startup, the uppermost WVC layer was inoculated with about 10 to 12 g/cm^3 of live worms ranging from 3 to 6 cm in length.

The crops for hydroponic growth were either offered by private growers or purchased locally at an Agriloja agricultural supply store. As the young plants had been growing in soil-filled cuvettes

at the moment of purchase, their roots were washed of soil before placing them in the net pots and securing with LECA.

2.3 Wastewater for treatment

The wastewater for vermifiltration/hydroponic treatment in the system was obtained from a piggery located in Leiria district. The piggery possesses a system of three sequential facultative lagoons for wastewater stabilization and initial treatment. For further treatment, wastewater is collected from the lagoons as they fill and transported in tanker vehicles to a large-scale WWTP located in the region. Samples were collected from the second lagoon as this should be representative of an intermediate stage of initial treatment. The wastewater was collected in 5-L plastic bottles for transportation. Larger solids were filtered on the spot through a 1.2-mm mesh colander; after transportation, additional solids were allowed to settle in the bottles before the water was decanted into the raw wastewater reservoir. A representative composition of wastewater from the same lagoon is presented in Table II.1 as determined previously (Pereira et al., 2019). Raw wastewater was diluted to 10% with water from the end reservoir in the proportion 9:1 (treated water : raw wastewater) or, to dilute the treated water as well, a proportion of 8:1:1 (treated water : clean water : raw wastewater) was used instead. The 10% dilution was chosen in order to initially decrease the electrical conductivity below levels reported to be toxic to *E. fetida*, LC_{50} = 0.183 S/m (Rahimi & Karimi, 2016).

2.4 Experimental timeline

The system was operated in four experimental periods, corresponding to treatments with different organism composition in the system.

Period 1, 59 days (December 5th, 2019, to February 2nd, 2020). Only the vermifilter was active in order to test and confirm some of its water treatment possibilities, widely documented in the literature (Elvira et al., 1996b; V. K. Garg et al., 2005, 2006; Gupta & Garg, 2008; Li et al., 2008).

Period 2, 45 days (February 3rd to March 19th). Introduction of the first plants into the hydroponic growth unit and their observation in order to choose the best surviving ones. Four different green leafy crops were planted in the hydroponic unit to be tested for their ability to adapt to hydroponic growth on vermifiltered piggery wastewater, 8 stocks of each: pointed cabbage (a *cultivar* of *Brassica oleracea*), loose-leaf lettuce (*Lactuca sativa*), spearmint (*Mentha*

spicata) and basil (*Ocimum basilicum*). The crops were fed by the vermifiltered water alone, without any supplementation with nutrients.

Between periods 2 and 3 there was a two-month gap due to external circumstances, when water circulation was interrupted, but aeration was kept active.

Period 3, 50 days (May 19th to July 8th). The previously selected leafy green was planted in all 40 positions of the hydroponic growth unit. The same circulating water from the previous phases was kept in order to provide nutrients to the plants. The hydroponically growing crop was fed by the vermifiltered water alone, without any nutrient supplementation.

Period 4, 41 days (July 11th to August 21st). The system was restarted after a complete cleanup with bleach, replacement of the vermifilter with a new one and replacement of the circulating water entirely with clean water. This was done in order to remove all micro-organisms that inhabited the whole system at that point and to observe the evolution of the conversion of different forms of nitrogen and phosphorus while the system was being repopulated. The plants were entirely replaced, this time by radicchio, or red chicory (*Cichorium intybus* var. *foliosum*). Water in the hydroponic tray was supplemented with a nutrient mixture based on the Hoagland solution nr. 1 (Hoagland & Arnon, 1950), where nitrates and phosphates were replaced with sulphates, calcium chloride and calcium carbonate (Appendix I, Table I.1). The mixture was prepared at a 100-fold concentration, and the supplementation was renewed weekly in two separate additions directly into the hydroponic tray, by replacing 1/200 of the tray liquid volume each time.

2.5 Hydraulic parameters

The approximate determination of hydraulic residence time (HRT) in the vermifilter and in the hydroponic tray, parameters relevant for the treatment efficiency assessment, was performed according to the equations:

$$HRT = \frac{V_L}{F}$$

(V_L = liquid volume in a compartment; F = liquid volumetric flow through the same compartment; A = feed contact area).

Volumetric flows through both compartments were calculated by measuring the volume of water entering and exiting the compartment during 10 to 15 minutes. Measurements were performed at entry and at exit to account for variations due to possible evaporation, leaf

evapotranspiration, or metabolic uptake or excretion of water by the different organisms present. Flow variation rate was considered independent of the entry and exit flow values, and thus a simple mean of those values was taken as the average flow through each compartment.

For the vermifilter, the maximum liquid volume at any time during treatment was considered to correspond to the void volume V_{void}, since at low volumetric flows it would not exert enough hydrostatic pressure to fill all gaps or displace the gases retained in the substrate pores. The void volume was determined by sealing the exit and lateral perforations on a filter identical to the used vermifilter (but without worms), filling it with a volume of water measured with graduated cylinders until the solids were covered, and then allowing the water to flow out, measuring its volume on exit (Garzón-Zúñiga et al., 2003).

For the hydroponic tray, the liquid volume was set at 120 L by the installed standpipe (Figure 2.4) when aeration was off. With the aeration on, the liquid volume V_{HP} was calculated as the difference between this maximum volume $V_{HP,max}$ and the average volume displaced by the floating rafts and aeration, $V_{HP,disp}$:

$$V_{HP} = V_{HP,max} - V_{HP,disp}$$

This displaced volume was estimated by the following procedure:

1) Floating rafts with plants were removed, the liquid flow and aeration were turned off, and the tray filled with water to the maximum level;
2) Then, the rafts placed back and aeration turned on, and the displaced water collected and measured on exit.

2.6 Hydroponic growth monitoring

The plants put to grow hydroponically from May 20th to July 7th (period 3) were monitored weekly for their growth and overall visual aspect reflecting their survival, health and nutrition. To each of the 40 plants corresponded a “chess board” position code (rows 1 to 8; columns A to E) (Figure 2.5). The following approximate measurements were performed with a tape measure: apparent stem length, calculated as stem length measured above the LECA level plus 2 cm to account for the estimated length covered by the LECA; apparent total aerial length, calculated as aerial length measured above the LECA level plus 2 cm; apparent leaf span diameter, measured as the widest observed leaf span perpendicularly to the stem, excluding the dead leaf tips when present. All the measurements were rounded to 0.5 cm. The number of leaves was counted considering all the observable leaves, excluding those with more than 50% dead area.

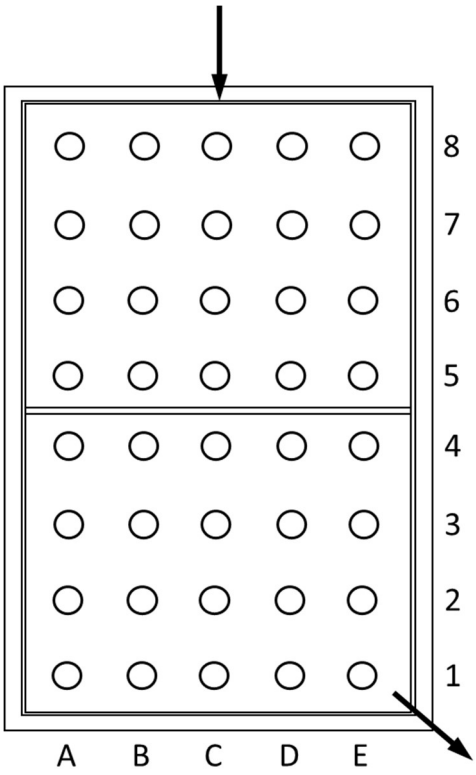

Figure 2.5: Schematic representation of the hydroponically cultivated plants position identification. The arrows indicate wastewater entry and exit points.

2.7 Micro-organisms

2.7.1 Coliforms and faecal streptococci

Coliforms were determined in water on different stages of treatment by the serial 10-fold dilutions method with filtration of known volumes of diluted sample through sterile Normax 0.45 µm pore diameter mixed cellulose esters (MCE) membranes, followed by inoculation on sterile Millipore Coliform ChromoSelect Agar plates, incubation at 37°C over 48 hours and counting of the coloured colonies on the suitable plates (dark blue or violet for *E. coli*, salmon to red for *Enterobacter cloacae* and *Citrobacter freundii*, and pink for *Klebsiella pneumoniae*) (Millipore, 2018).

Wastewater faecal streptococci were analysed by the serial 10-fold dilutions method with filtration of known volumes of diluted sample through sterile Normax 0.45 µm pore diameter mixed cellulose esters (MCE) membranes, inoculation on sterile Sigma-Aldrich Enterococcus Selective Agar (Slanetz-Bartley Agar) plates, incubation at 37°C for 48 hours and counting of the coloured colonies on the suitable plates.

All manipulations were performed under aseptic conditions. Standard error of the mean, or simply standard error (SE), was used as uncertainty measure in calculations. SE was defined as:

$$\mathrm{SE} = \frac{\mathrm{SD}}{\sqrt{n}}$$

(SD being the standard deviation, and *n* the number of replicas) (Miller & Miller, 2000).

2.7.2 Nitrogen-metabolizing bacteria

Commercial vermicompost (VC), earthworm-inhabited woodchips-vermicompost mixture (WVC), raw piggery wastewater (RWW) and water from different treatment stages were characterized qualitatively with respect to the ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) by Fluorescent In Situ Hybridization (FISH) techniques.

Vermicompost was previously rehydrated with clean water until moist but not flooded and left for 7 days at room temperature to reactivate the bacteria. Solid materials (VC and WVC) were suspended in phosphate-buffered saline (PBS) (0.01 mol/L phosphates, 0.13 mol/L NaCl, pH 7.2), at a ratio of 3 g solids : 2 mL PBS, with vigorous stirring. Raw wastewater was diluted 5 times with PBS and stirred vigorously. The obtained suspensions were filtered through Whatman Reeve Angel 0.8 – 0.9 µm pore diameter glass fibre filters to retain the larger solids but allow the bacteria through.

Water samples collected after 24 and 31 days of experimental period 4 from vermifilter feed, vermifilter effluent (hydroponic feed), the hydroponic tray and the treated water holding reservoir were concentrated by centrifugation and the pellets were resuspended in PBS.

Sample fixation for FISH analysis was based on standard protocols (Nielsen et al., 2009). The resulting suspensions were mixed with 3 volumes of 4% (m/v) parafomaldehyde, incubated on ice for 3 hours, centrifuged at 3500 g for 10 minutes, resuspended in ice-cold 1:1 PBS:ethanol mixture and frozen at –20°C (Nielsen et al., 2009).

For the detection of any active bacteria, fluorescein isothiocyanate (FITC)-labelled EUBmix (EUB338 (Amann et al., 1990), EUB338II and EUB338III (Daims et al., 1999)) oligonucleotide probes were used. For particular bacterial groups, the following cyanine 3 (Cy3)-labelled probes were applied: Nso1225 and Nso190 for betaproteobacterial ammonia-oxidizing bacteria (AOB) (Mobarry et al., 1996) and, for nitrite-oxidizing bacteria (NOB), NIT3 (*Nitrobacter spp*.) (Mobarry et al., 1996) and Ntspa662 (*Nitrospira spp*.) (Daims et al., 2001). Biomass was visualized under a

Zeiss Imager D2 epifluorescent microscope at 1000 X magnification. More information on oligonucleotide probes is available at probeBase 2016 (Greuter et al., 2016).

2.8 Water physical and chemical analysis

Samples were collected for analysis at several points throughout the system, on the day following the addition of a new portion of piggery effluent, diluted with treated water as described: from the vermifilter feed; at the vermifilter exit, which also represented hydroponic unit feed; and from the hydroponic growth container.

The collected water samples were analysed for the following physical and chemical parameters: electrical conductivity (EC), pH, total suspended solids (TSS) and total dissolved solids (TDS), 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total nitrogen (TN), nitrate, nitrite, ammonia nitrogen, total phosphorus (TP) and dissolved phosphorus.

All determinations were performed in triplicate for each sample, unless stated otherwise. For all colorimetric determinations, samples were previously filtered through Whatman Reeve Angel 0.8 – 0.9 µm pore diameter glass fibre filters, followed by Neoreax or Normax 0.45 µm pore diameter cellulose acetate or mixed cellulose esters (MCE) membranes to remove suspended solids.

Relative change (*RC*) of each parameter through treatment in each stage was calculated using the formula:

$$RC = \frac{x_{eft} - x_{feed}}{x_{feed}} \times 100\%$$

where x_{feed} represents a parameter value in the feed, and x_{eft} is the corresponding value in the effluent in each treatment stage.

Standard error of the mean, or simply standard error (SE), was used as uncertainty measure in all calculations.

To test the significance of changes due to the treatment, an analysis of variance (ANOVA) was performed. *P*-values indicate the probability of the null hypothesis to be true (parameter values at the end of a treatment being equal to those at the start). Significance was considered low for $P > 0.05$ and very low for $P > 0.50$.

2.8.1 Electrical conductivity and pH

Sample electrical conductivity and pH were measured directly by electrometric equipment. EC was measured by an Edge HI2030 conductometer connected to an HI763100 cell (Hanna Instruments). For pH measurements, a pH212 potentiometer with an HI1131 selective hydrogen electrode was used (Hanna Instruments).

2.8.2 Solids

Total suspended solids (TSS), total dissolved solids (TDS) and total solids (TS) were quantified according to the Standard Methods for Examination of Water and Wastewater (SMEWW) (APHA-AWWA-WEF, 2005).

Total suspended solids (TSS) were determined by filtering appropriate volumes *V* of previously stirred sample through Whatman Reeve Angel 0.8 – 0.9 µm pore diameter glass fibre filters (previously dried in an oven at 105°C to constant mass and weighed; mass registered as m_{ftr}); the filters were dried in an oven at 105 °C to constant mass, and their mass was again registered as $m_{ftr+sol}$. TSS content was calculated by the formula:

$$TSS(\mathrm{mg/L}) = \frac{m_{ftr+sol}(\mathrm{mg}) - m_{ftr}(\mathrm{mg})}{V(\mathrm{L})}$$

Briefly, for total dissolved solids (TDS) appropriate volumes *V* of filtered sample were transferred into porcelain capsules (which were previously dried in an oven at 180°C to constant mass and weighted; mass registered as m_{cap}) and evaporated in an oven at 105°C, again to constant mass. Masses of full (m_{full}) and evaporated (m_{evap}) capsules were registered. Sample TDS content was calculated as mass percentage by the formula

$$TDS(\%) = \frac{m_{evap}(\mathrm{g}) - m_{cap}(\mathrm{g})}{m_{full}(\mathrm{g}) - m_{cap}(\mathrm{g})} \times 100\%$$

and as mass concentration, by the formula

$$TDS(\mathrm{mg/L}) = \frac{m_{evap}(\mathrm{mg}) - m_{cap}(\mathrm{mg})}{V(\mathrm{L})}$$

Total solids (TS) content was then calculated as the sum of TS and TSS:

$$TS(\mathrm{mg/L}) = TDS(\mathrm{mg/L}) + TSS(\mathrm{mg/L})$$

2.8.3 Chemical and biochemical oxygen demand

Chemical oxygen demand (COD) corresponds to the total content of oxidizable organic matter in a sample, expressed as equivalent amount (mg) of O_2 needed for the oxidation, per litre. The so-called 5-day biochemical oxygen demand (BOD_5) corresponds to the organic matter that is oxidizable by aerobic biological activity over a 5-day incubation period, as equivalent milligrams of O_2 per litre.

COD was determined by open reflux / differential titration method according to the International Standard ISO 6060:1989 (ISO, 1989). 10.00 mL of appropriately diluted samples, blanks or control standard were incubated at 150°C for 2 h with 5.00 mL of potassium dichromate (0.0042 mol/L):mercury sulphate (0.027 mol/L) and 15.00 mL of 0.032 mol/L Ag_2SO_4 in concentrated sulphuric acid as catalyst, followed by titration with ferric ammonium sulphate (FAS) approx. 0.12 mol/L against a blank containing no sample. The titrant was previously standardized by titration of potassium dichromate:mercury sulphate solution; the exact FAS concentration was obtained by the formula:

$$[\mathrm{FAS}] = \frac{5 \times 0.042 \times 6}{V_{FAS}}$$

Where V_{FAS} is the volume of FAS solution added as titrant.

After titration with FAS, COD was calculated by the formula:

$$COD(\mathrm{mgO_2/L}) = \frac{8000 \times [\mathrm{FAS}](V_1 - V_2)}{V_{spl}}$$

(V_1 = titrant volume used on blank, V_2 = titrant volume used on the sample, V_{spl} = sample volume)

BOD_5 was determined in water samples according to the International Standard ISO 5815-1:2019 (ISO, 2019). Dilution water was buffered to pH 7.2 with phosphate buffer and contained 0.0086 mol/L allylthiourea (for nitrification inhibition), 0.0913 mol/L magnesium sulphate, 0.248 mol/L calcium chloride, 0.000925 mol/L iron (III) chloride and, for pH 7.2 phosphate buffer, 0.062 mol/L potassium dihydrogen phosphate, 0.125 mol/L potassium hydrogen phosphate, 0.125 mol/L sodium hydrogen phosphate, and 0.032 mol/L ammonium chloride. Appropriately diluted samples were placed in glass Winkler bottles, and dissolved oxygen was measured with a YSI 5000 dissolved oxygen meter connected to a YSI 5010 selective probe. Then the bottles were water-sealed by glass stoppers and incubated at 26(±1)°C for 5 days, after which the dissolved oxygen was again measured. The decrease in dissolved oxygen concentration (mg/L) after the 5-day incubation was considered to correspond to BOD_5.

2.8.4 Nitrogen

Total Nitrogen

Total nitrogen content was determined as nitrate after alkaline digestion with potassium persulphate, according to SMEWW 4550-N C (APHA-AWWA-WEF, 2005). Nitrate was quantified after digestion of appropriately diluted samples (adjusted to pH = 7) with 0.025 mol/L potassium persulphate and 0.025 mol/L NaOH for 55 min at 110°C (JP Selecta Micro 8 bench autoclave), which converted all forms of nitrogen into nitrate. Nitrate was then determined by the brucine colorimetric method according to EPA 352.1 Method (EPA, 1971). Appropriately diluted samples were allowed to react with 0.62 mmol/L brucine sulphate : 0.33 mol/L sulphanilic acid in ~6,5 mol/L sulphuric acid at 100°C for 25 min, and absorbance was read at 410 nm (Varian Cary 50 Conc UV-visible spectrophotometer). Potassium nitrate was used as standard for calibration curves.

Nitrate

Aqueous nitrates were determined potentiometrically according to SMEWW 4500-NO_3^- D or colorimetrically according to SMEWW 4550-N C (APHA-AWWA-WEF, 2005). Potassium nitrate was used as standard for calibration curves. For potentiometric determination, appropriately diluted samples, standards and blank and mixed with buffered (pH = 3) reagent solution, containing 0.013 mol/L aluminium sulphate, 0.005 mol/L silver sulphate, 0,010 mol/L boric acid and 0.013 mol/l sulphamic acid (final concentrations in the mixture), and potential was read with an HI 3221 pH/ORP/ISE Meter with an HI 4113-51 Nitrate Combination Electrode (Hanna Instruments). Colorimetric determination followed the procedure described above for total nitrogen, excluding the digestion steps and after filtration as described.

Nitrite

Nitrite was determined colorimetrically according to SMEWW 4500-NO_2^- B (APHA-AWWA-WEF, 2005). Calibration curves were produced using sodium nitrite as standard. Samples, filtered as described for nitrate, were diluted by the adequate dilution factor with ultrapure water, and their pH was adjusted when necessary to fall within the range from 5 to 9, with either 1 mol/L ammonium hydroxide or 1 mol/L hydrochloric acid. Diluted samples, standards or blank, were incubated at room temperature with a colouring reagent (2.2 mmol/L sulphanylamide and 0.15 mmol/L *N*-(1-naphthyl)-ethylenediamine dihydrochloride) for at least 10 min, and absorbance was read at 543 nm (Varian Cary 50 Conc UV-visible spectrophotometer).

Ammonia

Ammonia (NH_4^+/NH_3) was determined colorimetrically according to the international standard ISO 7150-1:1984 (ISO, 1984).Calibration curves were made with ammonium chloride as standard. Standards, blanks, or appropriately diluted samples were incubated at 25(±1)°C for 1 hour with 0.065 mol/L sodium salicylate, 0.035 mol/L trisodium citrate, 0.26 mmol/L sodium nitroprussiate, and 0.86 mmol/L sodium dichloroisocyanurate at pH 12.8. Absorbance was measured at 655 nm (Varian Cary 50 Conc UV-visible spectrophotometer).

2.8.5 Phosphorus

Total and dissolved phosphorus were determined colorimetrically according to SMEWW-P E (APHA-AWWA-WEF, 2005). To quantify dissolved phosphorus, samples were previously filtered as described. For total phosphorus determination, samples were digested by adding 1 mL of concentrated sulphuric acid and 5 mL of concentrated nitric acid to 50 mL of sample and heating until volume decreased to 10 mL; then 10 mL of ultrapure water were added, and the mixture was neutralized with 1 mol/L NaOH, using phenolphthalein as indicator. Then, in both procedures, colorimetric quantification was performed, using a calibration curve obtained with dihydrogen phosphate as standard. Volumes of 50.00 mL of diluted samples, standards or blanks were incubated at room temperature for 10 to 30 min with 8.00 mL of a mix of 5 mol/L sulphuric acid, 0.43 mmol/L sodium antimonium tartrate, 4.9 mmol/L ammonium molybdate and 0.03 mol/L ascorbic acid. Absorbance was read at 880 nm (Varian Cary 50 Conc UV-visible spectrophotometer).

In all colorimetric methods, which required calibration curves, the standard concentrations were defined previously to present a good linearity (determination coefficients higher than 0.99) over an interval where a diluted or undiluted sample could fall. Sample concentration values were considered below the limit of quantification (LOQ) when undiluted samples yielded absorbance values below the first non-zero standard.

3. Results and discussion

3.1 Wastewater flow and hydraulic residence times

The determination of hydraulic residence time (HRT) in a trickling filter, such as the vermifilter used, is complicated since it depends on the flow and hydraulic pressure, which dictate the filling of voids and pores and, thus, the effective volume occupied by the liquid (Garzón-Zúñiga et al., 2003).

The maximum HRT for the vermifilter was calculated as the ratio between the vermifilter void volume $V_{VF,void}$ and the liquid volumetric flow F. Void volume was calculated as described, resulting $V_{VF,void}$ = 4.4 L. Thus, the maximum hydraulic residence time was thus estimated to be

$$HRT = \frac{V_{VF,void}}{F} = \frac{4.4\ \text{L}}{11\ \text{L/day}} = 0.4\ \text{days} \approx 10\ \text{h}$$

Similar vermifilters have been reported to best operate at HRT of 6 to 10 h (Lourenço & Nunes, 2017b).

HRT for the hydroponic unit corresponded to the ration between the total volume of the liquid in it and the volumetric flow. The total (maximum) liquid volume was measured as 120(±3) L. The displaced and the remaining liquid volumes were calculated as described in Methods:

V_{disp} = 6(±0.2) L

$V_{HP} = V_{HP,max} - V_{HP,disp}$ = 120(±3) L – 6.4(±0.2) L ≈ 114(±3) L

The hydraulic residence time in the hydroponic unit was thus estimated to be, on average:

$$HRT = \frac{V_{HP}}{F} \approx \frac{114}{11} = 10.4\ \text{days}$$

These values were valid when the peristaltic pump was working properly; failure to pump was, however, sometimes observed due to larger solid particles trapped in the pump hose. This would increase the average HRT both in the vermilfilter and in the hydroponic tray.

HRT has a non-linear effect on treatment efficiency in continuous systems. Shorter HRT only give the organisms in each compartment the opportunity to remediate the wastewater to a limited extent; HRT cannot be too long either since that would imply very large volumes or very slow flows and little throughput. Long HRT complicate the calculation of treatment efficiencies, as

will be shown later. Complications that arose due to HRT in this study would be eliminated by using batch systems instead of continuous ones.

Other advantages of using batch systems would be the ability to independently test different piggery wastewater dilutions, treated wastewater recirculation rates, to correct pH and electrical conductivity to specific values and to test different crops for survival, growth and treatment efficiency.

The idea, however, was to build and test a working continuously fed system as could be implemented in real-life pig farm conditions. For all the experienced difficulties, the studied system provided some useful information, as will be discussed later.

3.2 Preliminary vermifiltration treatment results (periods 1 and 2)

During the first experimental period, the vermifilter was operating without the added hydroponic treatment. This served the purpose of allowing the earthworms and microbial community to adapt to the pig farm wastewater in the feed while observing and correcting flaws in the construction and hydraulic behaviour of the system. Some analyses were performed during this period to confirm the existing information on vermifiltration wastewater treatment and to know what to expect of it before introducing hydroponic plants. The main focus of this study was placed on later experiments, on nitrogen and phosphorus remediation by hydroponic cultures when fed on vermifiltered wastewater with recirculation of the final effluent. Wastewater from the same swine farm had been previously characterized at LSRE-LCM Leiria pole (Pereira et al., 2019); the data are shown in Appendix II.

All physical, chemical and biological quantitative data are presented as mean ± standard error (SE) (Miller & Miller, 2000).

3.2.1 Colour and odour

Vermifiltration showed effects on the swine wastewater smell and colour. Even 10-fold diluted wastewater presented a characteristic foul odour before vermifiltration; after vermifiltration, no noticeable odours remained. Throughout the treatment, a continuous change in vermifiltered water colour towards darker shades of brown was observed over time. This may have been related to the production of humic substances, which are brown-coloured polymeric organic compounds (Gerke, 2018; Kosobucki & Buszewski, 2014), as has been reported to

happen during vermifiltration in several studies (Elvira et al., 1996b; Pereira et al., 2014; Yang et al., 2014).

3.2.2 Solids

Total suspended solids (TSS) and total dissolved solids (TDS) content were determined at the start and at the end of vermifiltration treatment on two separate dates. The results are presented in Table 3.1. According to the results, total suspended solids were significantly removed from the wastewater, while total dissolved solids did not show a significant change until after 39 days. A month later, after 67 days, TDS content increased significantly in the effluent, which may reflect the increased mineralization due to of heterotrophic organisms. Vermifiltration was able to reduce TSS but not TDS content below the maximum recommended values according to Portuguese law for irrigation water (60 mg/L TSS, 640 mg/L TDS) and wastewaters discharge into the environment (60 mg/L TSS) (Ministério do Ambiente, 1997, 1998).

These results must be seen critically since TSS in the vermifilter effluent showed large standard errors, which translated into high uncertainties associated with relative change. This may be due to some smaller suspended particulate matter, which passes through the vermifilter together with water, being prone to some chemical or biological degradation and thus able to produce greatly varying analyses. Better sample preservation during analysis could improve this situation, and it should be useful to use larger replica numbers than the triplicate that was used. Nevertheless, the results confirm the information available in literature (T. Kumar et al., 2014; Lourenço & Nunes, 2017a; Manyuchi et al., 2013; Sinha et al., 2014). The use of triplicates was chosen as the reasonable approach to all assays for practical reasons of time and laboratory material saving.

Table 3.1: Total suspended solids (TSS) and total dissolved solids (TDS) content and relative change by vermifiltration treatment alone, as mean(±SE).

t (days)	Parameter	Feed (mg/L)	Effluent (mg/L)	Rel. change (%)	*P*-value
39	TSS	61.7(±0.9)	30(±5)	−52(±8)	0.00265
	TDS	$1.22(\pm0.03)\times10^3$	$1.29(\pm0.04)\times10^3$	+6(±4)	0.254
67	TSS	71(±3)	34(±11)	−52(±17)	0.0363
	TDS	$1.29(\pm0.04)\times10^3$	$1.80(\pm0.02)\times10^3$	+40(±3)	<0.001

3.2.3 Electrical conductivity and pH

Electrical conductivity (EC) and pH were measured in the feed of the vermifilter in order to control whether the feed was adequate for the earthworms. They were also measured in the effluent from the vermifilter to see whether the treatment altered either of these parameters, affecting water quality for hydroponic cultivation. According to Portuguese legislation, the pH of irrigation waters must fall between 4.5 and 9.0, recommended range from 6.5 to 8.4 (Ministério do Ambiente, 1998). Soil EC for leafy horticultural crops such as cabbage, celery and lettuce has tolerance thresholds between 0.1 S/m and 0.2 S/m (Jarwal et al., 2006). In hydroponic systems, EC tolerance is higher, but still an EC higher than 0.2 S/m has been reported to negatively affect crops growth (Wortman, 2015). On the other hand, high EC has been shown to have negative effects of on survival and growth of *Eisenia fetida*, with a 50% lethal dosage of 0.183 S/m (Rahimi & Karimi, 2016).

The results of EC and pH measurements are presented in Table 3.2. Both the electrical conductivity and pH showed values within the acceptable ranges for earthworms and hydroponic crops. EC, like the directly related TDS content, did not show a significant increase that could be expected as a result of organic matter degradation by earthworms and commensal bacteria and as has been reported to happen (P. Garg et al., 2006; Gupta & Garg, 2008), although other studies suggested differently (V. K. Garg et al., 2006).

Table 3.2: Electrical conductivity and pH in vermifilter feed and effluent measured at 41 days of period 1, as mean(±SE).

Parameter	Feed	Effluent	Relative change (%)	*P*-value
EC (S/m)	0.154 (±0.001)	0.151(±0.003)	−2(±2)	0.396
pH	6.60±0.01	6.75±0.01	+2.3(±0.2)	<0.001

3.2.4 COD and BOD_5

Organic matter was quantified as COD and BOD_5. COD was used to represent the total (oxidizable) organic matter present in the samples and its chemical or biological degradation in the process, whereas BOD_5 specifically represented biodegradable organic matter. The results are shown in Table 3.3.

The results suggest a good biologically available organic matter removal and a less efficient total organic matter removal by vermifiltration. Literature refers to removals of 98 to 99% BOD_5, and 70 to 92% COD by vermifiltration of sewage and dairy industry effluents (Manyuchi et al., 2013;

Sinha et al., 2007). As observed from the darkening water colour and also reported in literature, part of the organic matter may have been converted to humic substances (Elvira et al., 1996b; Pereira et al., 2014; Yang et al., 2014), which would contribute to COD but much less to BOD due to their recognised resistance to biodegradation (Gerke, 2018; Kosobucki & Buszewski, 2014). Since the vermifilter works at the same time as a mechanical filter operating by gravity and as a biological reactor, organic matter removal can be a combination of biological digestion and mineralization of organic substances and physical removal of particulate matter of larger size by filtration.

Table 3.3: COD and BOD_5 and the corresponding relative change caused by vermifiltration treatment alone at 41 days of period 1, as mean(±SE).

Parameter	Feed (mgO_2/L)	Effluent (mgO_2/L)	Relative change (%)	*P*-value
COD	228(±7)	155(±6)	−32(±4)	0.0014
BOD_5	41.3(±1.1)	7.0(±0.4)	−83(±4)	<0.001

3.2.4 Nitrogen

Total nitrogen, as well as different forms of inorganic nitrogen relevant from the point of view of nitrogen cycle reaction were quantified. The results are shown in

Table 3.4, Table 3.5, Table 3.6 and Table 3.7. The results suggest that there has been a significant occurrence of change in each of the four parameters determined, although ammonia and nitrite in vermifilter effluent and relative changes showed low or very low precision (high uncertainty). This, once again, shows the variability and unpredictability of samples involving biological activity. High uncertainty associated with values suggests the need to use larger sample sizes (e.g. quadruplicates) in order to decrease standard errors and, if necessary, discard outlying values. Additionally, physical or chemical inhibition methods might be used to arrest all biological activity while collecting and preparing samples for analysis; however, this could increase time and monetary cost of these analyses. Quick cooling, like placing samples on ice, should be the cheapest way to achieve this goal.

The observed variations suggest a highly efficient removal of ammonia nitrogen: in all instances, the effluent ammonia was below the limit of quantification (LOQ) at the highest possible sample concentration during analysis. These results are in agreement with the observations published by numerous authors (Li et al., 2008; Villar et al., 2016; Wang et al., 2013). The removal of nitrites was a little less efficient; nitrates, on the contrary, showed an increase, resulting from a balance

between nitrification and denitrification activities. Nitrite has been reported in some publications to increase during earthworm treatment of solid waste and sewage (Rajpal et al., 2014); in the present case, if the NOB activity is higher than that of AOB, it would be expected to decrease. Nitrate content has been reported in the literature to increase due to earthworm activity on sewage sludge (Yang et al., 2014). The overall change in different nitrogen forms content was favourable to the use of vermifiltration effluent for hydroponic crop cultivation, since the treatment showed a potential ability to enrich water in nitrates. Total nitrogen did not show a significant change throughout the process. The vermifilter was designed as a chemically open system, continuously fed with wastewater containing high amounts of ammonia and organic nitrogen as products of swine metabolism. Besides, not only within the vermifilter itself but also in the feed container and in the collected effluent, all processes of organic nitrogen transformation can occur: ammonification of organic matter, nitrification of ammonia to nitrite and then nitrate, nitrogen incorporation in organic matter as worms and bacteria grow, and some of the nitrogen would be inevitably lost as N_2 due to bacterial denitrification. Therefore, relative removal or accumulation of different forms of nitrogen is more relevant than their absolute values in the feed or the effluent. The increase in nitrate did not fully account for the overall ammonia and nitrite removal, as should be expected when denitrification and biological assimilation are also present.

Table 3.4: Total nitrogen content and relative change caused by vermifiltration treatment alone (41 days), as mean(±SE).

t (days)	Feed (mg N/L)	Effluent (mg N/L)	Relative change (%)	*P*-value
41	111(±1)	107(±1)	-3(±1)	0.043

Table 3.5: Ammonia nitrogen (NH_3-N) content and relative change caused by vermifiltration treatment alone, as mean(±SE). LOQ: limit of quantification.

t (days)	Feed (mg NH_3-N/L)	Effluent (mg NH_3-N/L)	Relative change (%)	*P*-value
41	7.32(±0.17)	<LOQ	−100	<0.001
68	39.6(±0.2)	<LOQ	−100	<0.001
96	17.8(±0.1)	<LOQ	−100	<0.001

Table 3.6: Nitrite (NO_2-N) content and relative change caused by vermifiltration treatment alone, as mean(±SE). LOQ: limit of quantification.

t (days)	Feed (mg NO_2-N/L)	Effluent (mg NO_2-N/L)	Relative change (%)	*P*-value
41	16.8(±0.1)	3.7(±1.2)	−78(±7)	<0.001
68	0.14(±0.01)	<LOQ	−100	<0.001
96	0.981(±0.007)	0.0131(±0.0002)	−99(±1)	<0.001

Table 3.7: Nitrate (NO_3-N) content and relative change caused by vermifiltration treatment alone, as mean(±SE).

t (days)	Feed (mg NO_3-N/L)	Effluent (mg NO_3-N/L)	Relative change (%)	*P*-value
41	50.2(±1.5)	64.4(±0.6)	+28(±3)	<0.001
68	192(±6)	318(±4)	+65(±4)	<0.001
96	57(±2)	69.7(±1.9)	+22(±5)	0.014

3.2.5 Phosphorus

Total and inorganic phosphorus were quantified in the vermifilter feed and effluent, and the results are presented in Table 3.8. Total phosphorus was significantly more abundant than dissolved phosphorus both in the feed ($P = 0.010$) and the effluent of the vermifilter ($P = 0.015$), as expected. Both total and dissolved phosphorus concentration showed changes with high calculated uncertainty and/or low significance; moreover, dissolved phosphorus showed changes in either direction (increase or decrease) in repeated analyses. Therefore, there does not seem to be a consistent and significant phosphorus removal or production by this biological treatment.

Table 3.8: Total and dissolved phosphorus content and relative change caused by vermifiltration treatment alone, as mean(±SE).

t (days)	Parameter	Feed (mg P/L)	Effluent (mg P/L)	Relative change (%)	*P*-value
41	P_{total}	6(±1)	5.0(±0.3)	−17(±18)	0.40
	$P_{dissolved}$	4.9(±0.3)	4.7(±0.4)	−3(±10)	0.78
68	$P_{dissolved}$	5.46(±0.01)	5.32(±0.04)	−2.6(±0.7)	0.025
89	$P_{dissolved}$	42.6(±0.15)	45.4(±0.4)	+6.6(±0.9)	0.0019
96	$P_{dissolved}$	22(±2)	22.7(±0.9)	+2(±12)	0.84

3.2 Plant selection (period 2)

The most promising adaptation and growth was observed for the cabbage, having displayed noticeable growth and healthy leaf colour and texture (see Figure 2.2). Lettuce showed some growth but also quickly showed leaf yellowing, indicating nutrient deficiencies (Marulanda & Izquierdo, 1993); spearmint and basil were not able to survive. Thus, the cabbage was chosen as the crop to be tested for wastewater treatment.

3.3 Complete vermifilter/hydroponic system (periods 3 and 4)

The initial treatment tests by vermifiltration alone largely confirmed the results obtained previously by several authors, as discussed above. Next, the treatment resulting from the combination of a vermifilter with a hydroponic growth unit in a single system with effluent recirculation needed to be tested as well. Here, the main focus was on the assessment of physico-chemical (electrical conductivity, pH, BOD_5, nitrogen and phosphorus) and biological parameters (faecal coliforms, faecal streptococci, AOB and NOB), as well as growth and well-being of the plants.

3.3.1 Behaviour of the hydroponically growing crops

Based on the observations of crop survival in the system, as mentioned above, the pointed cabbage was chosen as the crop to be used in hydroponic water treatment tests. Cabbage seedlings were planted in all the 40 pots of the hydroponic growth unit (Figure 2.5). The plants showed measurable growth of aerial parts length and horizontal spread, stem height, and number of leaves (Figure 3.1). Visual analysis also showed problems due to deficiencies in nutrients such as, at least, iron and magnesium: yellowing of younger leaves between veins, and potassium: leaves dying at the tips, leaves curling (Marulanda & Izquierdo, 1993) (Figure 3.2). The results suggest that diluted and vermifiltered piggery wastewater as was used in this study cannot provide all the necessary nutrients for plant growth and development. Quantitative analysis of such wastewaters for the essential nutrients and supplementation with those below the necessary levels could be essential to allow for growth of healthy, nutritious crops for human or animal consumption, and also the metabolic capacity to efficiently remove the target pollutants: inorganic nitrogen and phosphorus.

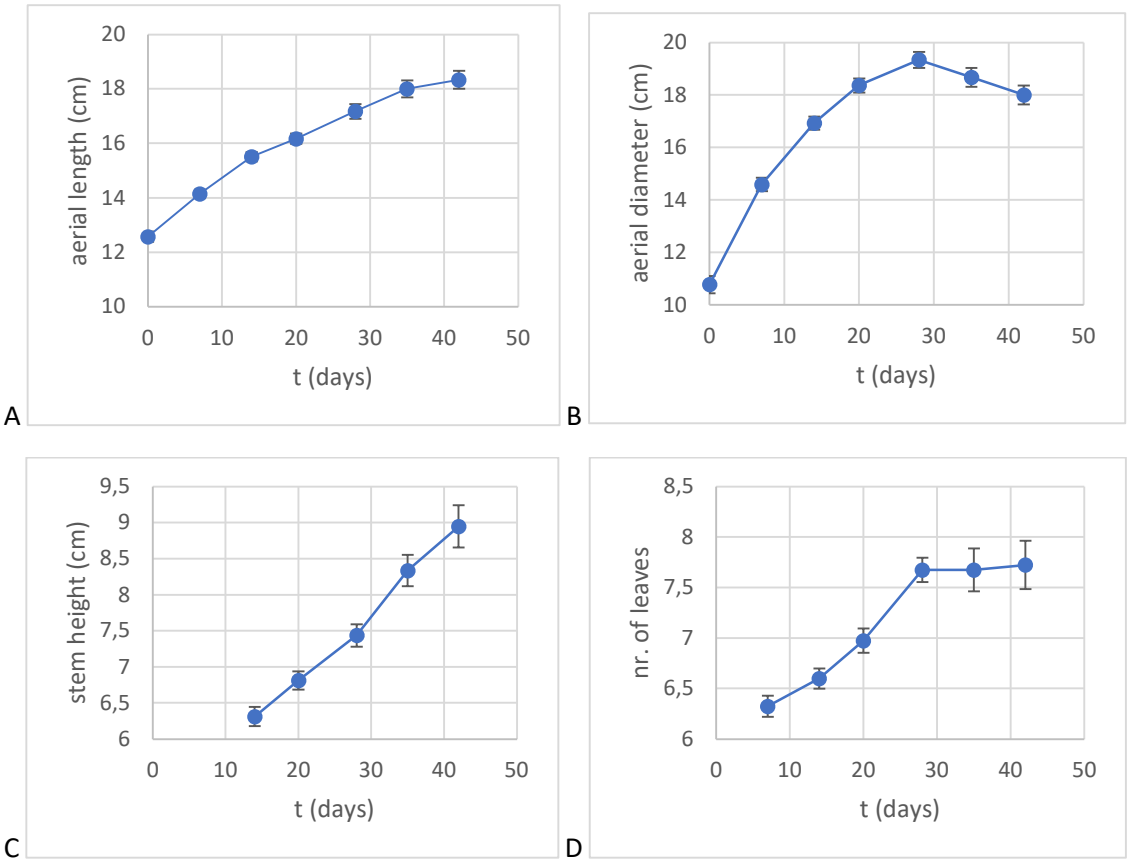

Figure 3.1. Growth of hydroponically cultivated crops during the experimental period 3: aerial parts length (A), aerial parts spread (B), stem height (C) and number of leaves (D).

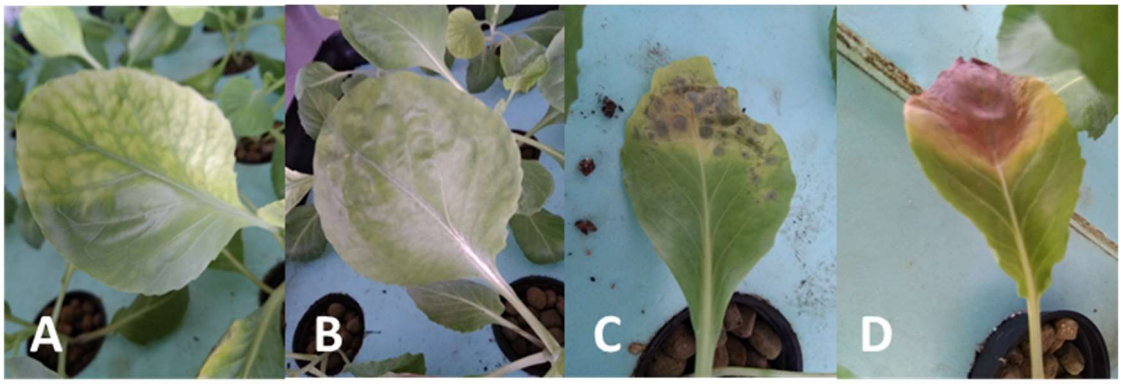

Figure 3.2. Evidence of nutrient deficiency observed on cabbage leaves. A: leaf yellowing in-between veins; B: leaf blade wrinkling; C and D: blade tip necrosis.

The position on the hydroponic rafts, referenced from A1 to E8 like on a chess board, was not shown to have any influence on each plant's growth and health. Stem and global aerial parts growth, as well as the increase of number of leaves during growth or its decrease due to nutritional deficiencies and other possible problems, did not correlate with the plants' position (Figure 3.3, Figure 3.4). These results agree with the assumption of a high degree of

homogeneity in the hydroponic tray due to aeration mixing and suggest that each plant's individual physiology, rather than position, may have influenced their development.

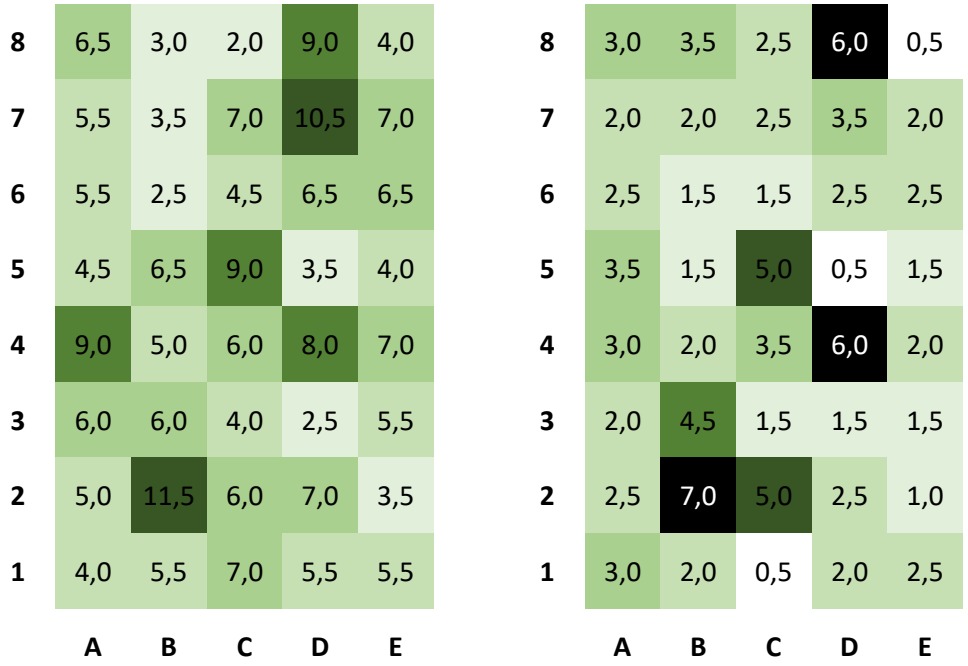

Figure 3.3: Individual increase in total aerial length (cm, left) and stem length (cm, right) throughout the cabbage seedlings growth period. Darker colour corresponds to greater growth.

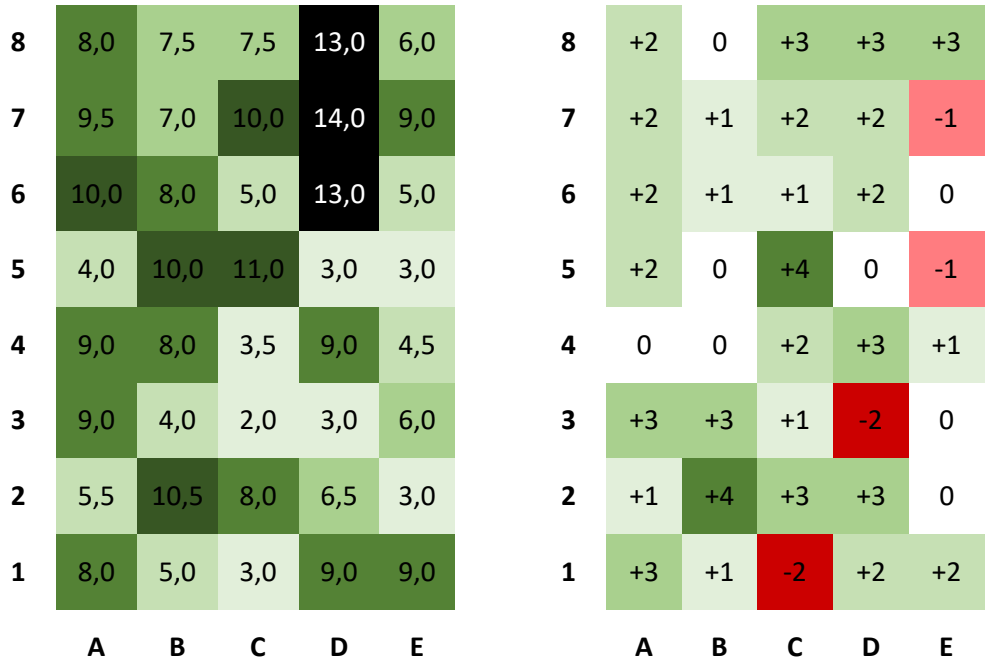

Figure 3.4: Individual increase in leaf span (cm, left) and gain or loss of leaves (right) throughout the cabbage seedlings growth period. Darker green to black colour corresponds to greater growth. Red spectrum colours correspond to overall loss of leaves.

After about seven weeks of pointed cabbage growth, the plants were showing signs of accelerated deterioration, with few to no live leaves left, and thus were discarded. At this point, the system was disinfected to remove all micro-organisms, and the circulating water fully renovated. This time, radicchio (*Chicorium intybus*) seedlings were planted in all 40 positions. The change of species from previously used cabbage to radicchio was due to different seasonal availability of both species seedling on the market. This time, a 100-fold concentrated nutritious solution based on Hoagland solution (Hoagland & Arnon, 1950) with omission of nitrogen and phosphorus was prepared to supplement the hydroponic water in an attempt to keep the plants healthier and improve nitrogen and phosphorus removal.

During period 4, radicchio plants showed no measurable growth, and every week some plants presented dying leaves. On the fifth week, it was noticed that several plants were dying and three completely dead.

3.3.2 Faecal coliforms and streptococci

Faecal coliforms and streptococci were analysed by CFU counting after a month (day 31) of experimental period 4. 0.300-mL volumes of undiluted samples were used. Coliform analysis suggested a reduction of these pathogens by 54% by vermifiltration. Elimination of coliforms by vermifiltration has been reported before (Edwards & Fletcher, 1988; Lourenço & Nunes, 2017a; Yadav et al., 2010). However, their content increased by 56% in the hydroponic effluent than in the vermifilter effluent and increased further by 55% in the treated water reservoir (Figure 3.5, Figure 3.6). This suggests that the subsequent hydroponic treatment did not contribute to further remove coliforms and can serve as a warning that using coliform-contaminated wastewaters for hydroponic growth can provide good conditions for their proliferation and spread, and increase the risk of a more serious contamination of the final effluent.

Faecal streptococci analysis did not provide conclusive results. Colonies were detected in undiluted vermifilter feed in numbers lower than 10, which is below the acceptable for quantification by CFU counting. No colonies were observed in vermifilter effluent, hydroponic effluent or final reservoir water.

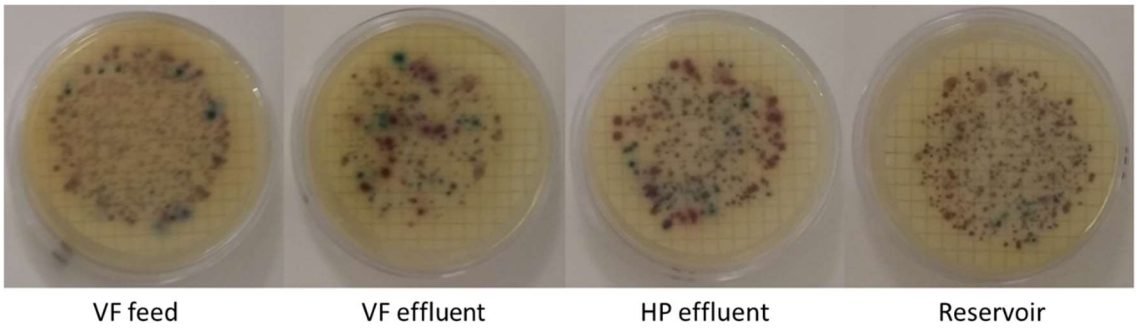

Figure 3.5: Representative coliform identification agar plates after inoculation of 0.300 mL and incubation.

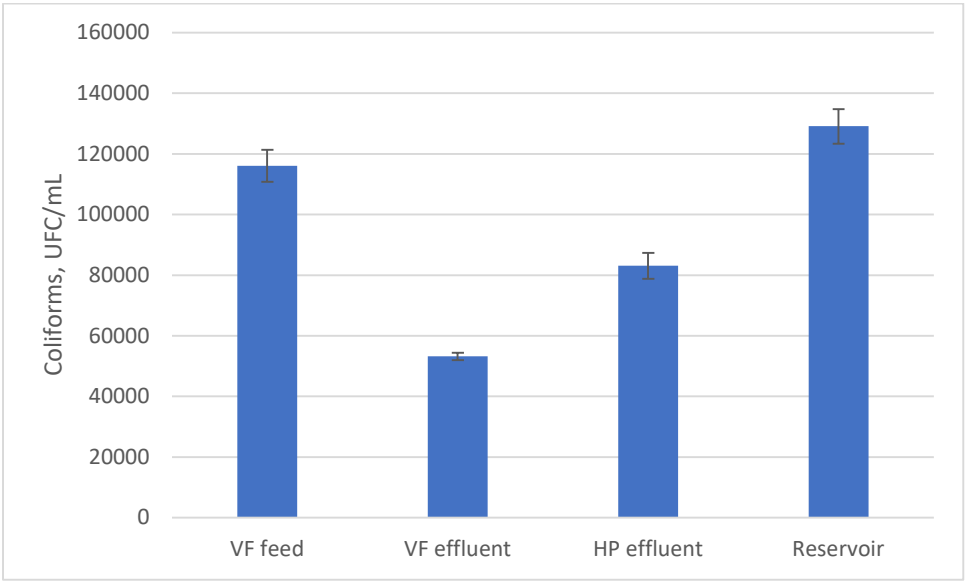

Figure 3.6: Total coliforms in wastewater at different process stages.

3.3.3 Nitrogen-metabolizing bacteria

The results of the AOB and NOB relative abundance analysis by FISH are presented in Table 3.9 and Figure 3.7. The observations did not reveal any active biomass collected from RWW, VC and WVC samples. The absence of microbial biomass in those samples may have resulted from the initial filtration through 0.45-µm pore membranes, removing the bacteria along with solids. It was not possible, due to time constraints, to repeat the assays with a different procedure.

In water samples, which had not been filtered, general bacterial biomass and different levels of AOB and NOB abundance were observed. Betaproteobacterial AOB Nso190 signal was consistently more intense than that of Nso1225. These bacteria were observed to be abundant in the vermifilter feed, showing both bacterial aggregates and single rod-shaped cells. Their abundance decreased to lower levels in the following treatment stages, with mostly free rod- and coccoid-shaped cells showing positive hybridization; the numbers were somewhat higher in the post-hydroponic reservoir. NOB also showed a higher abundance in the vermifilter feed than

in the vermifilter effluent (hydroponic feed), hydroponic effluent and post-hydroponic reservoir; no evident aggregates were observed. *Nitrospira* probe Ntspa662 showed a consistently lower signal than *Nitrobacter* probe NIT3, which may suggest a stronger presence of *Nitrobacter spp*. Lower abundance of both metabolically active AOB and NOB in the liquid phase after the vermifiltration stage may result from attachment to solids and biofilm colonization inside the vermifilter; nitrifying bacteria have been shown to display complex plankton-biofilm distribution and interactions with other bacterial groups in nature and in water treatment systems (Del'Duca et al., 2019; Soliman & Eldyasti, 2018). It should be noticed that the AOB and NOB abundance in biofilms adhering to the hydroponic tray internal surface, LECA and plant roots was not accounted for and must be addressed in further studies.

Table 3.9: Qualitative assessment of AOB and NOB abundance in water at different collection points. Cells detected with specific probes were classified as non-existent (–), present (+), abundant (++) or dominant (+++).

Time (days)	**Sample collection point**	**AOB**		**NOB**	
				Nitrospira	***Nitrobacter***
		Nso1225	Nso190	Ntspa662	NIT3
24	VF feed	**++**	**++**	**+**	**+**
	VF effluent / HP feed	**–/+**	**+/++**	**–**	**+**
	HP effluent	**–/+**	**+**	**–**	**–/+**
	Reservoir	**–/+**	**+**	**–**	**–/+**
31	VF feed	**++**	**++**	**+**	**+/++**
	VF effluent / HP feed	**–/+**	**+**	**–/+**	**–/+**
	HP effluent	**–**	**+/++**	**–**	**–/+**
	Reservoir	**–**	**+/++**	**–/+**	**–/+**

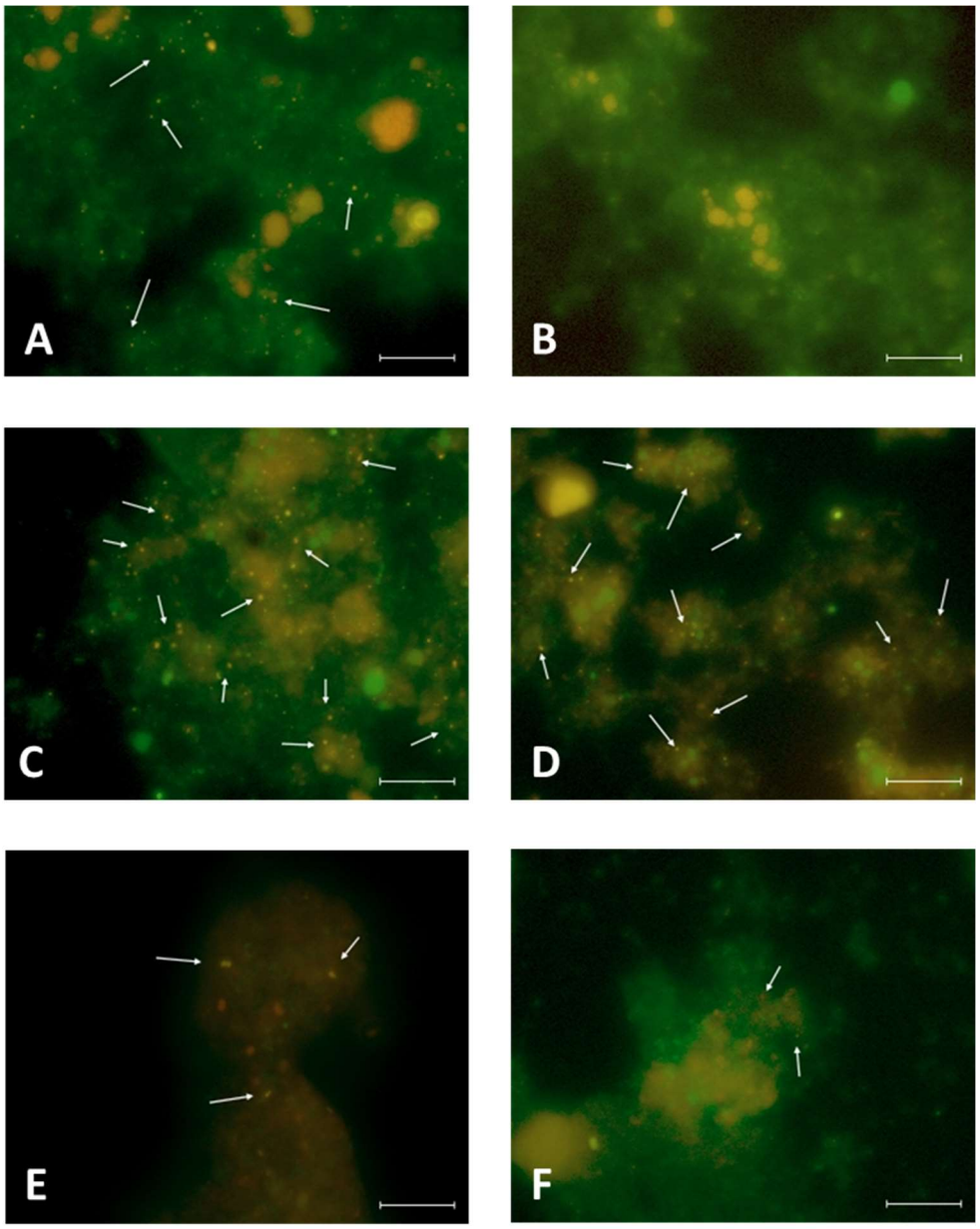

*Figure 3.7: Examples of AOB and NOB relative abundance in FISH images. Total cell biomass is stained green (FITC-labelled EUBmix probe), and AOB or NOB are stained red. Yellow/orange results from the overlay of both colours. The examples are: abundant (**A**: VF feed at 24 days, Nso1225 probe; **B**: VF feed at 31 days, Nso190 probe), present (**C**: VF feed at 24 days, Ntspa662 probe; **D**: VF feed at 24 days, NIT3 probe), and almost non-existent (**E**: VF effluent at 24 days, Nso1225 probe; **F**: VF effluent at 31 days, Ntspa662 probe). Arrows indicate examples of single cells. Scale bars = 20 µm.*

3.3.4 Physico-chemical analysis

The results of piggery wastewater treatment by vermifiltration and subsequent hydroponic treatment were obtained from samples collected at the piggery facilities from December 2019 to July 2020. Solids content, electrical conductivity, pH, organic matter (COD and BOD_5), different chemicals forms of nitrogen and phosphorus were determined by standard analytical methods (see Material and Methods section) in wastewater fed to each compartment (vermifilter and hydroponic tray) and in the treated water exiting the compartment, which will be referred to as effluent, in order to evaluate the relative change of each parameter occurring at this stage of biological treatment.

This principle was applied to the vermifilter and the hydroponic treatment stage, considering the hydraulic residence times. New raw wastewater was always introduced into the mixing tank at least 18 hours prior to sample collection for analysis. If the maximum HRT in the vermifilter was 10 h, it was considered reasonable to compare the vermifilter effluent directly to the feed sample collected on the same morning. A more likely estimate for the vermifilter HRT should be 5 to 6 h.

For hydroponic treatment, the situation was more complex. The hydraulic residence time was estimated to be of approximately 10.5 days, which meant that, on average, every component spent that time in the hydroponic tray while being or not converted into a product. The analyses were performed at most weekly due to time, equipment and reagent limitations. New portions of raw wastewater, which certainly underwent some alterations while staying in the reservoir, were also added weekly, about 18 h prior to the collection; this, minus the vermifilter HRT (circa 6 h), would be approximately 12 h, or 0.5 days. Besides, since at least 80% of the final treated wastewater was returned into the mixing tank, the hydroponic effluent inevitably affected the composition of the vermifilter feed. Since the HRT was estimated to be of 10.4-10.5 days, which is close to the average of one and two weeks, the best estimate of the hydroponic feed parameters could only be obtained by averaging feed parameter values from one and two weeks prior to each effluent value, as those were the closest known values characterizing the feed on each occasion. A much more reliable way to assess hydroponic treatment would have been to work in batch conditions; however, the idea was to test a system working continuously, as it had been conceived for possible implementation in the field. Thus, the best estimate of the representative feed composition over the 10.5 days in the hydroponic unit was considered to be the weighted mean of feed from 14 days before $x_{feed}(t–14)$ over 3 days, feed from 7 days

before $x_{feed}(t–7)$ over 7 days, and feed collected on the same day as effluent $x_{feed}(t)$ over the remaining 0.5 days.

The following approximation was used to calculate the relative changes in water quality parameters due to the hydroponic treatment:

$$RC \approx \frac{x_{eft}(t) - \left[\frac{3x_{feed}(t-14) + 7x_{feed}(t-7) + 0.5x_{feed}(t)}{10.5}\right]}{\left[\frac{3x_{feed}(t-14) + 7x_{feed}(t-7) + 0.5x_{feed}(t)}{10.5}\right]} \times 100\%$$

This approach accounts for the average permanence of a solute inside the hydroponic tray, although it does assume a constant vermifilter effluent composition over the entire 7 days between new wastewater additions. It also demands additional days of analyses to account for the delay. In order to assess the hydroponic treatment more accurately, batch treatment with a known feed composition or a continuous system with constant feed composition would be a better solution.

Under real conditions such a system, operating over extensive time periods, is likely to suffer malfunctions. This was indeed observed, most noticeably as clogging of the vermifilter feed line with solids from the raw wastewater samples. Although the samples had been strained and decanted before entering the system, still some solid particles were large enough to compromise the operation of the peristaltic pump and stop the feed flow for an undetermined time, probably for hours. Inside the vermilfilter, substances were produced by the earthworms as vermicast, and microbial aggregates formed. These components altered the granulometry and chemical nature and, presumably, density, water content and viscosity of the solid substrate, altering its water permeability. As was previously observed by other co-workers (L. Pereira & L. Aires, unpublished data), this could ultimately compromise the vermifilter function by causing flooding and hypoxic conditions.

It should be noticed that the “weighted mean” used for hydroponic feed values calculation was not a statistical mean of equal independent values, but an estimate of a certain component’s concentration resulting from three different inputs. Therefore, the associated standard deviation and standard error were calculated by uncertainty propagation from the standard deviations and standard errors of the three contributing triplicates, scaled to match the weighted mean.

The experimental periods 3 and 4 were conducted under different conditions concerning the initial liquid phase composition. In period 3, the treated wastewater from periods 1 and 2 was kept as the sole nutrient source for plants; in period 4, the system was decontaminated from micro-organisms and started on clean dechlorinated tap water, supplemented with nutrients based on Hoagland's nr. 1 solution, as explained in Material and Methods. These two different approaches were followed in order to compare the treatment by plants starting from Hoagland's solution and changing the composition of the circulating treated water over time with plants fed on "mature" treated wastewater, with a presumably established microbial community and stabilized concentrations of certain accumulated nutrients. The former scenario was investigated to observe the evolution of hydroponic treatment of nitrogen and phosphorus pollution. The latter one is closer to what would be expected from a wastewater treatment system implemented at a real piggery.

Electrical conductivity and pH

Electrical conductivity and pH values, measured over time in the feed, the vermifilter (VF) effluent and the hydroponic unit (HP) effluent during experimental period 3, are presented in Table 3.10. This time, in contrast with the results obtained with a vermifilter alone, an increase in electrical conductivity was observed, most noticeably from the feed to the effluent of the vermifilter at 21 days of the experimental period 3. The reason for a much lower EC in the feed than in both other samples on that day is unclear. Subsequent measurements showed relatively high EC values till 42 days. pH showed neutral to slightly alkaline values, higher in the vermifilter effluent and hydroponic effluent.

During the experimental period 4, even higher EC and pH values were measured (Table 3.11). During that period, the system was supplemented with nutrients; however, the increased EC could not be assigned to the supplementation, since it never exceeded the Hoagland's solution concentrations, and the nutrient mixture EC was measured to be 0.108 S/m at that maximum concentration. The nutrient solution pH was 7.7, which is slightly alkaline, but still does not account for final values above 8.

According to a 2016 technical report produced at Oklahoma State University, EC ranging from 0.25 S/m to 0.30 S/m should be optimal for cabbage (unspecified variety); optimal pH was reported to be between 6.5 and 7.0 (Singh & Dunn, 2016). On the other hand, increased salinity has been reported to impair nitrogen, potassium, calcium and magnesium leaf concentrations in Chinese cabbage (Lira et al., 2015), so some metabolic damage on the crops might still result.

For *Eisenia fetida*, negative effects of high EC on survival and growth were reported, the 50% lethal dosage after 42 days being 0.183 S/m (Rahimi & Karimi, 2016). Alkaline pH promotes the deprotonation of ammonia to its toxic form NH_3, which might also affect the earthworms; however, a study of both ammonia and pH increase on a vermifiltration system reported little to no negative effects on the operation (Hughes et al., 2008). Alkaline pH has also been reported to impair nitrite oxidation activity in polluted waters (Le et al., 2019).

Table 3.10: EC and pH single readings in the complete combined system during period 3.

	EC (S/m)			pH		
Time (days)	**VF Feed**	**VF effluent/ HP feed**	**HP effluent**	**VF Feed**	**VF effluent/ HP feed**	**HP effluent**
21	0.1610	0.3050	0.2928	6.72	7.34	7.65
28	0.2237	0.2413	0.2776	7.38	7.60	7.77
35	0.2475	0.2517	0.2989	7.02	7.61	7.77
42	0.2901	0.2940	0.2642	6.52	7.25	7.69
49	0.1591	0.1635	0.1605	6.87	7.71	7.71

Table 3.11: EC and pH as mean(±SE) in the complete combined system during period 4.

	EC (S/m)			pH		
Time (days)	**VF Feed**	**VF effluent/ HP feed**	**HP effluent**	**VF Feed**	**VF effluent/ HP feed**	**HP effluent**
32	0.3503 (±0.0012)	0.3393 (±0.0017)	0.341 (±0.002)	7.74 (±0.00)	7.65 (±0.04)	8.45 (±0.04)
39	0.3920 (±0.0000)	0.3877 (±0.0015)	0.3777 (±0.0019)	7.82 (±0.01)	7.80 (±0.03)	8.38 (±0.02)

Biochemical oxygen demand

BOD_5 was determined in period 3, when it was supposed that the liquid medium, enriched in organic matter from the previous processes, could allow for a sensitive analysis of its removal in both vermifilter and hydroponic unit.

Over the four-week period, the vermifiltration showed the ability to remove from 66 to 83% of BOD_5 (Table 3.12), consistently with previous results and with observations reported in the literature for various wastes (Lourenço & Nunes, 2017b, 2017a; Manyuchi et al., 2013; Rajpal et al., 2014; Sinha et al., 2007). The removal of 83(±2)% at 42 days was identical to that observed in preliminary analysis at 41 days of the vermilfilter operating alone, 83(±4)%. According to Portuguese law, the maximum allowed value for discharge from a wastewater treatment plant

is 25 mgO_2/L, and removal efficiency should be at least 70% (Ministério do Ambiente, 1997). The observed removal of BOD_5 by vermifiltration generally met the legal requirements.

Table 3.12: BOD_5 and relative change as mean(±SE) in the vermifiltration unit of the complete combined system during period 3.

Time (days)	Feed BOD_5 (mgO_2/L)	Effluent BOD_5 (mgO_2/L)	Relative change (%)	*P*-value
28	24.4(±0.7)[a]	5.4(±0.4)	–78(±4)	<0.001
35	15.4(±0.4)	5.9(±0.3)	–66(±3)	<0.001
42	35.1(±0.3)	5.8(±0.3)	–83(±2)	<0.001
49	31(±1)	6.1(±0.3)	–80(±4)	<0.001

[a]One of three replicas was rejected

BOD_5 determination in the hydroponic feed and effluent provided a view on the fluctuations of the composition of wastewater under treatment, related to numerous factors such as temperature, dissolved oxygen concentration, other solutes and different organisms' activity. It was possible to measure the change in BOD_5 at two time points. Unaveraged HP feed and effluent values are shown in Appendix III: Table III.1. Values treated by the weighted average approach are shown in Table 3.13. On both occasions, a significant decrease of organic matter measured as BOD_5 was observed. Thus, the addition of a hydroponic treatment stage further improved the removal of organic matter from swine farm wastewater.

Table 3.13: BOD_5 and relative change as mean(±SE) in the hydroponic unit of the combined system during period 3.

Time (days)	Feed BOD_5, three dates weighted average (mgO_2/L)	Effluent BOD_5 (mgO_2/L)	Relative change (%)	*P*-value
42	5.7(±0.6)	3.01(±0.09)	–47(±12)	<0.001
49	5.9(±0.7)	2.17(±0.08)	–63(±15)	<0.001

Nitrogen

Similarly to the results from the initial assays without a coupled hydroponic unit, the vermifiltration-hydroponic system showed the capacity to significantly eliminate ammonia. The relative changes during period 3 (Table 3.14, Figure 3.8) were always negative, although in two instances the decrease was lower, by 15(±3)% and 20(±8)% (the latter value being associated with a somewhat lower significance, $P = 0.057$). This lower removal efficiency occurred on the days of lowest feed ammonia content, which might mean that less ammonia could be removed before attaining the balance between nitrification and other contributing factors such as ammonia excretion by earthworms. Feed ammonia content varied substantially, possibly due to

heterogeneity in the stored sample, which was not stirred to better represent the variable composition of real piggery wastewaters. During period 4 (Table 3.15, Figure 3.9), vermifiltration was able to remove from 98 to 100% of ammonia. Efficient ammonia nitrogen removal by vermifiltration has been reported in the literature (Li et al., 2008; Lourenço & Nunes, 2017b, 2017a; Wang et al., 2013).

Table 3.14: Ammonia nitrogen (NH_3-N) content and relative change, as mean(±SE), in the vermifiltration unit of the complete combined system during period 3. LOQ: limit of quantification.

Time (days)	Feed (mg NH_3-N/L)	Effluent (mg NH_3-N/L)	Relative change (%)	*P*-value
0	5.66(±0.06)	0.342(±0.009)	–94(±1)	<0.001
7	0.69(±0.09)	0.154(±0.007)	–78(±17)	0.0048
21	0.100(±0.003)	0.0852(±0.0011)	–15(±3)	0.010
28	8.9(±0.5)	0.075(±0.004)	–99(±7)	<0.001
42	0.088(±0.003)	0.070(±0.006)	–20(±8)	0.057
49	2.67(±0.01)	<LOQ	–100	<0.001

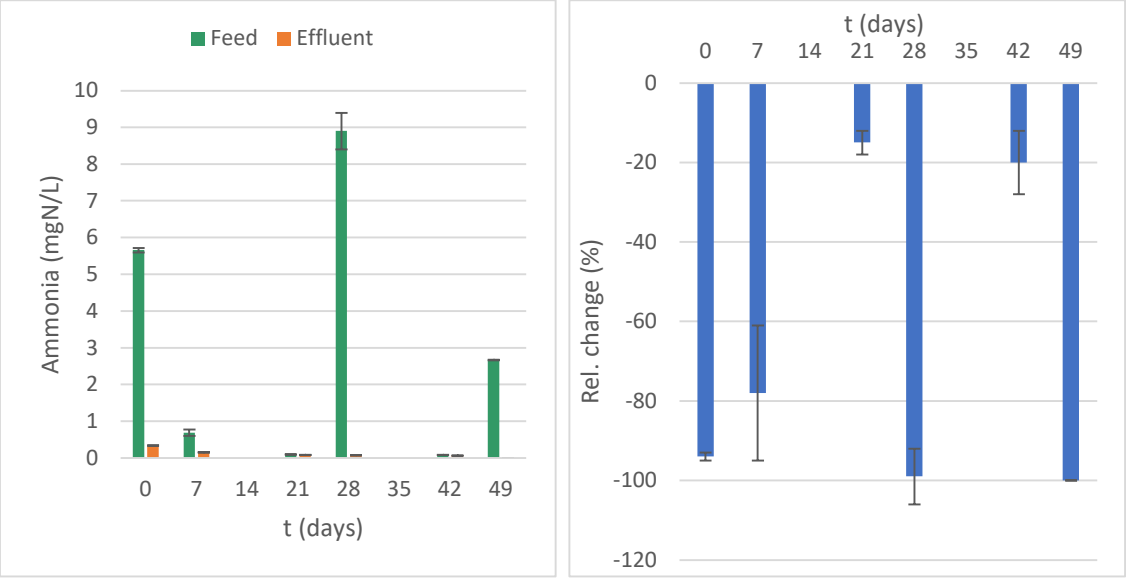

Figure 3.8: Ammonia nitrogen (NH_3-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), due to vermifiltration over time during period 3. SE is represented by bars.

Table 3.15: Ammonia nitrogen (NH_3-N) content and relative change, as mean(±SE), in the vermifiltration unit of the complete combined system during period 4.

Time (days)	Feed (mg NH_3-N/L)	Effluent (mg NH_3-N/L)	Relative change (%)	*P*-value
18	36.8(±1.0)	0.090(±0.002)	–100(±4)	<0.001
25	25.8(±0.4)	0.0958(±0.0011)	–99.6(±1.9)	<0.001
32	10.3(±0.2)	0.181(±0.011)	–98(±3)	<0.001
39	15.3(±0.1)	0.124(±0.002)	–99.3(±0.1)	<0.001

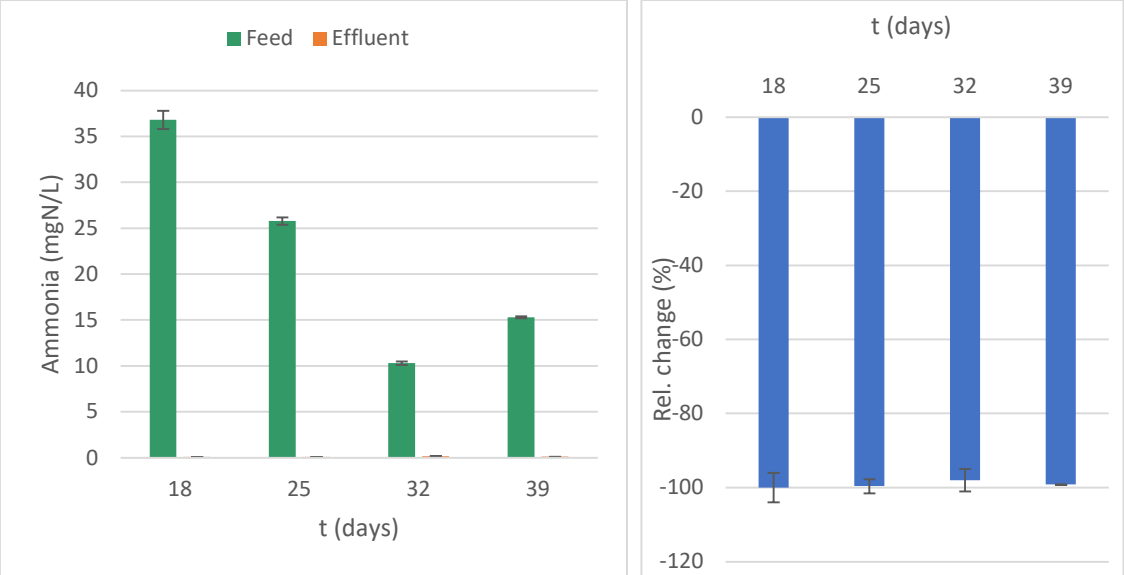

Figure 3.9: Ammonia nitrogen (NH_3-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), due to vermifiltration over time during period 4. SE is represented by bars.

Concerning ammonia content changes during the hydroponic treatment, the starting concentrations were already very low after vermifiltration. Further reduction was observed in the hydroponic unit, sometimes leading to ammonia levels below the limit of quantification (LOQ) of the method, in undiluted samples. The HP feed and effluent values obtained over time during period 3 are presented in Table 3.16. Period 4 unaveraged HP feed and effluent values can be seen in Appendix III: Table III.2. Averaged feed, effluent and relative change during period 4 are presented and in Table 3.17 and Figure 3.10. The results suggest activity of ammonia-oxidizing micro-organisms in the hydroponic unit, which was expectable since that unit was fed with vermifilter effluent, enriched in such micro-organisms, and the hydroponic tray was aerated, creating the necessary aerobic conditions. During period 3 the values in the effluent fell below the LOQ at after 28 days; thus, it was not possible to quantify the removal by the weighted average approach. It has to be considered that feed ammonia concentration was low, also falling below the LOQ at 49 days. During period 4, it was possible to estimate ammonia removals by almost 100% after 25 days. Ammonia uptake activity was consistent with the presence of active AOB in the vermifilter feed, detected by FISH analysis. As discussed above, part of those AOB may have colonized the solids and joined the biofilm communities in the vermifilter, since less AOB were found in the vermifilter effluent. Less significant changes in ammonia were estimated when the hydroponic feed levels were already below the Portuguese legal limit for wastewater discharge of 8.2 mg NH_3-N/L (Ministério do Ambiente, 1998).

Table 3.16: Ammonia nitrogen (NH_3-N) content as mean(±SE) in the hydroponic unit feed and effluent during period 3.

Time (days)	VF effluent/HP feed (mg NH_3-N/L)	HP effluent (mg NH_3-N/L)
0	---------	---------
7	0.154(±0.007)	0.127(±0.012)
21	0.0852(±0.0011)	0.0726(±0.0019)
28	0.075(±0.004)	<LOQ
42	0.070(±0.006)	<LOQ
49	<LOQ	<LOQ

Table 3.17: Ammonia nitrogen (NH_3-N) content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mg NH_3-N/L)	Effluent (mg NH_3-N/L)	Relative change (%)	*P*-value
25	3.96(±0.04)	0.064(±0.003)	–98(±1)	<0.001
32	0.100(±0.003)	0.103(±0.001)	+3(±3)	0.186
39	0.15(±0.02)	0.099(±0.006)	–36(±16)	<0.001

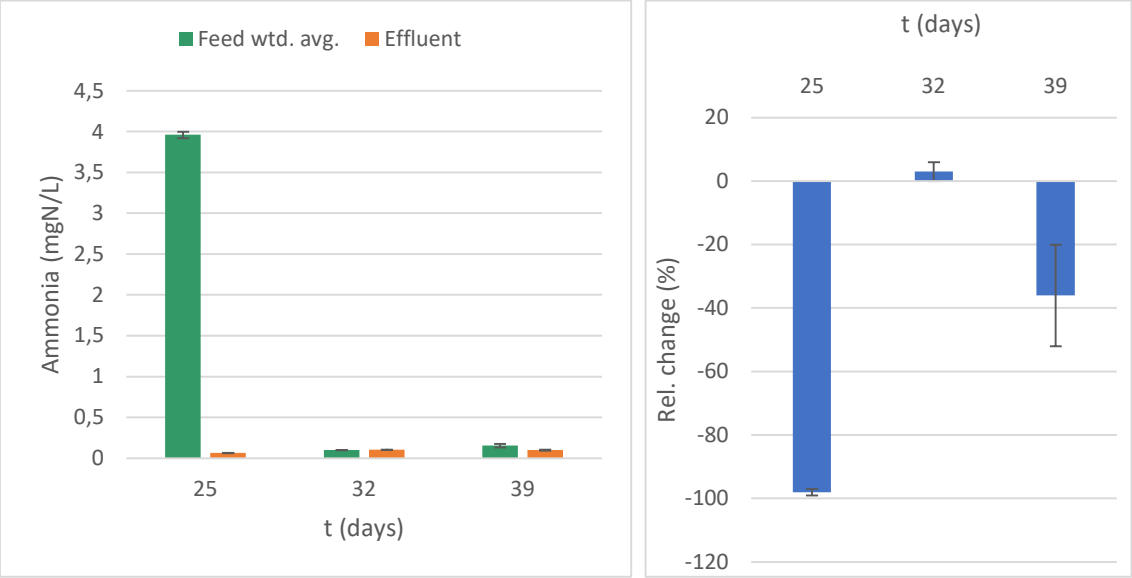

Figure 3.10: Ammonia nitrogen (NH_3-N) concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), due to hydroponic treatment over time during period 4. SE is represented by bars.

During period 3, vermifiltration treatment was able to cause decreases in nitrite concentrations to values below 0.1 mg NO_2-N/L (Table 3.18, Figure 3.11). Only in two moments the decrease was not observed, and that was when the feed values were particularly low from the start.

During period 4 (Table 3.19, Figure 3.12), removal efficiencies from 95 to 100% were observed, comparable to the efficiency of more sophisticated swine manure treatments such as anaerobic digesters combined with trickling filters (Terán et al., 2017) or immobilized cells reactors (Szögi et al., 2004). Nitrite removal was consistent with the previously discussed NOB detection in the vermifilter feed and their possible transfer from the liquid to the solid phase inside the vermifiler. Wastewater treatment by vermifiltration has been reported to favour nitrification processes (T. Kumar et al., 2014; Lourenço & Nunes, 2017a); present results support those assertions.

Table 3.18: Nitrite (NO_2-N) content and relative change as mean(±SE) in the vermifiltration unit of the combined system during period 3.

Time (days)	Feed (mg NO_2-N/L)	Effluent (mg NO_2-N/L)	Relative change (%)	*P*-value
0	8.31(±0.09)	0.0825(±0.0014)	–99(±2)	<0.001
7	0.0421(±0.0001)	0.0507(±0.0005)	+20.4(±1.3)	<0.001
28	2.19(±0.01)	0.0307(±0.0002)	–98.6(±0.4)	<0.001
35	0.174(±0.017)	0.0342(±0.0006)	–80(±12)	0.0011
42	0.0196(±0.0017)	0.0273(±0.0004)	+39(±10)	<0.001
49	4.58(±0.04)	0.0676(±0.0004)	–98.5(±1.3)	<0.001

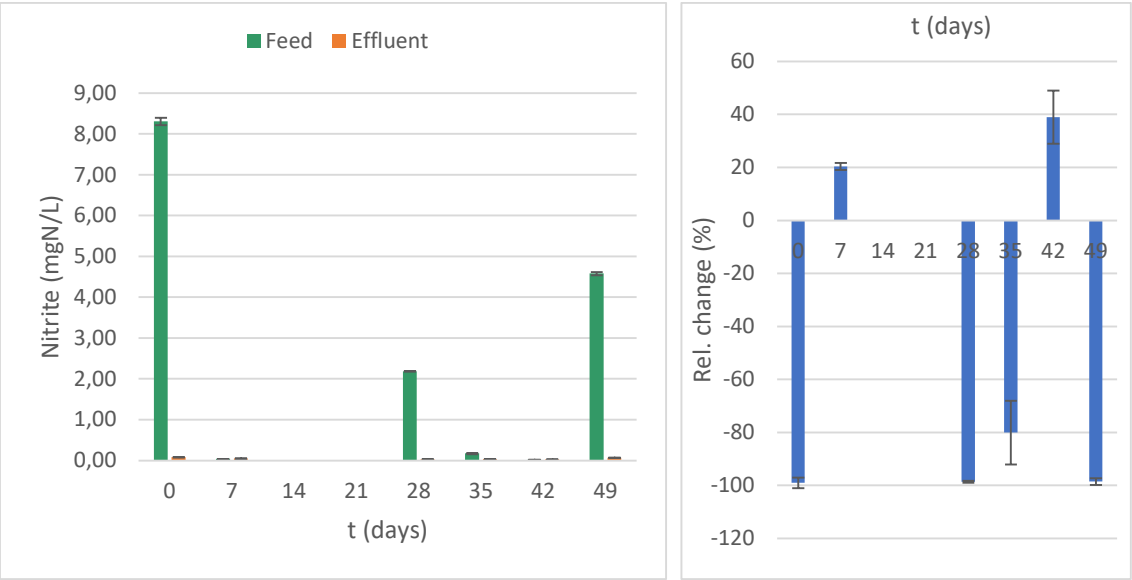

Figure 3.11: Nitrite (NO_2-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), due to vermifiltration over time during period 3. SE is represented by bars.

Table 3.19: Nitrite (NO_2-N) content and relative change as mean(±SE) in the vermifiltration unit of the combined system during period 4.

Time (days)	Feed (mg NO_2-N/L)	Effluent (mg NO_2-N/L)	Relative change (%)	*P*-value
18	38.8(±0.4)	1.83(±0.03)	–95.3(±1.3)	<0.001
25	43.6(±0.4)	0.0898(±0.0003)	–99.8(±1.4)	<0.001
32	47.6(±1.7)	0.0856(±0.0003)	–100(±5)	<0.001
39	2.16(±0.02)	0.0660(±0.0001)	–97(±1)	<0.001

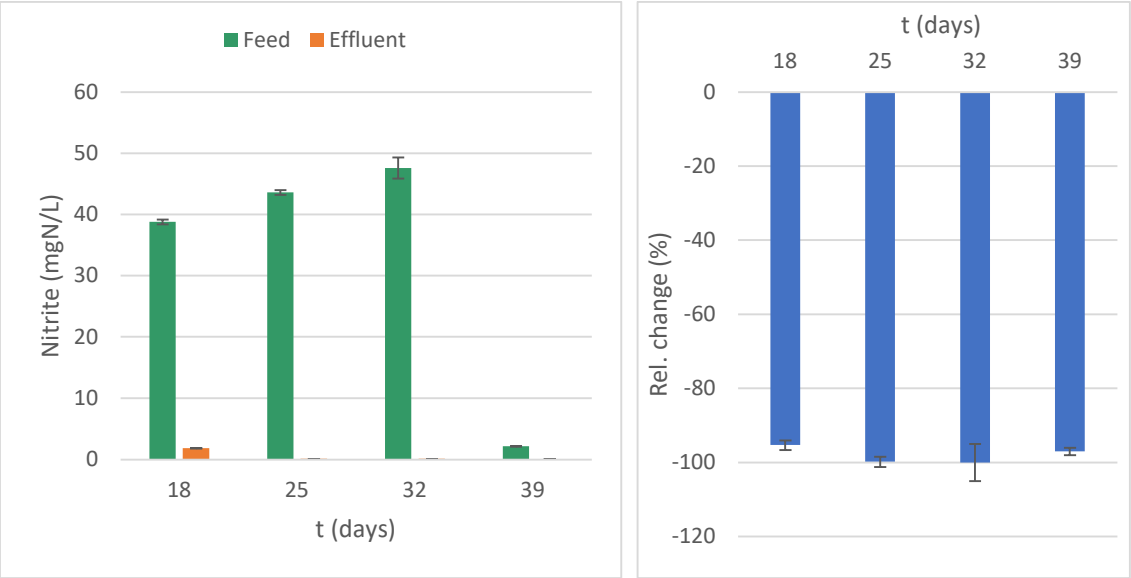

Figure 3.12: Nitrite (NO_2-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), due to vermifiltration over time during period 4. SE is represented by bars.

Nitrite averaged feed, effluent and relative change values are presented in Table 3.20 and Figure 3.13 for period 3, and in Table 3.21 and Figure 3.14 for period 4. Unaveraged values at each time point are shown in Appendix III: Table III.3 and Table III.4. Nitrite content showed a general decrease along the hydroponic treatment. Like with ammonia, nitrite decrease was expectable to happen due to the activity of nitrite-oxidizing micro-organisms transferred from the vermifilter by the liquid flow and proliferating in the hydroponic tray under the created aerobic conditions, probably mostly as part of biofilms. Removal efficiency by this treatment stage was lower than the achieved by vermifiltration in both experimental periods 3 and 4, and this could again be attributed to the lower starting values. Ecological balance of all the species involved in the nitrogen cycle requires the availability of their main nutrients on certain level, and for this reason all nutrients, including nitrite, should evolve towards certain sustained optimal levels. The presence and activity of nitrifying micro-organisms both in the liquid phase and in biofilms within the system should be more thoroughly investigated in the future.

Table 3.20: Nitrite (NO_2-N) content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 3.

Time (days)	Feed, three dates weighted average (mg NO_2-N/L)	Effluent (mg NO_2-N/L)	Relative change (%)	*P*-value
42	0.0329(±0.0013)	0.0112(±0.0012)	–66(±6)	<0.001
49	0.0312(±0.0011)	0.0208(±0.0001)	–33(±4)	<0.001

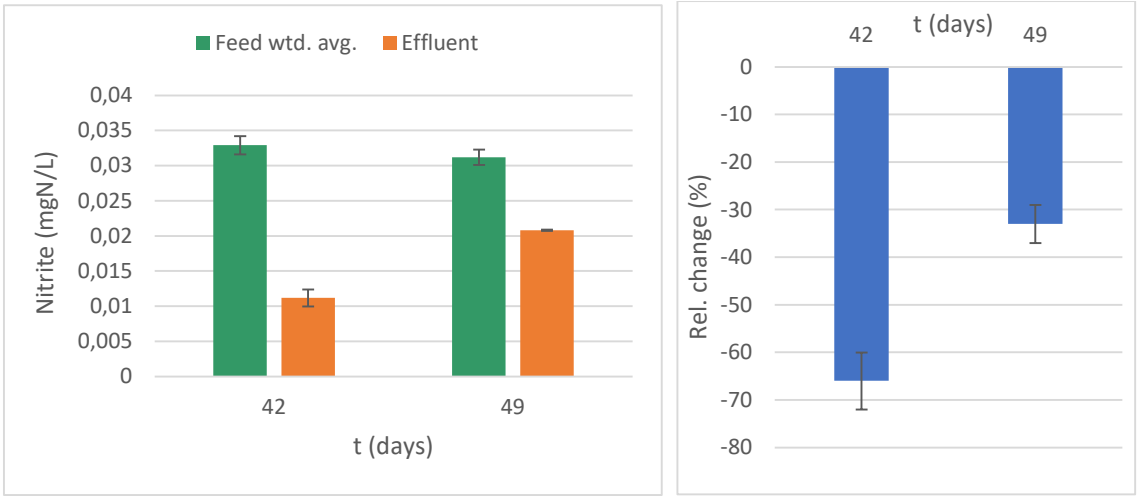

Figure 3.13: Nitrite (NO_2-N) concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 3. SE is represented by bars.

Table 3.21: Nitrite (NO_2-N) content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mg NO_2-N/L)	Effluent (mg NO_2-N/L)	Relative change (%)	*P*-value
25	3.79(±0.17)	0.0192(±0.0001)	–99(±6)	<0.001
32	0.59(±0.03)	0.0374(±0.0001)	–94(±6)	<0.001
39	0.0859(±0.0007)	0.0397(±0.0007)	–54(±1)	<0.001

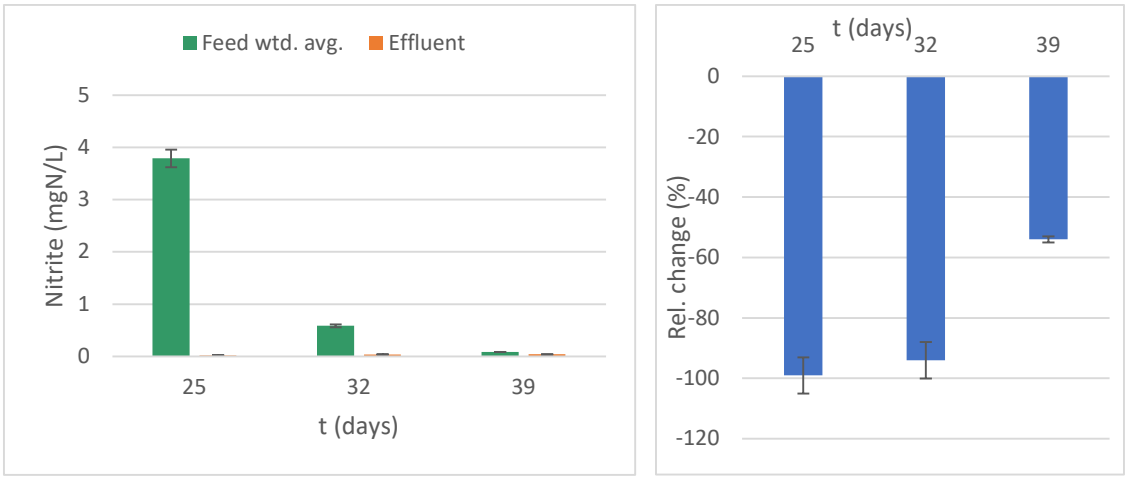

Figure 3.14: Nitrite (NO_2-N) concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

For nitrate, the results from the vermifilter feed, vermifilter effluent (hydroponic feed) and hydroponic effluent were all close to each other at several time points during period 3. This can be explained by the fact that 80% of the volume transferred to the mixing tank corresponded to the hydroponic effluent, gathered in the final reservoir for 7 days. The close values in different treatment phases at each time point can be explained by the micro-organisms spread through the system and the fact that most of the treated water was returned to the mixing tank along with every new sample addition. Little to no relative increase in nitrate was observed during that period (Table 3.22, Figure 3.15). During period 4, earlier values were lower than those of period 3, but a tendency to increase over time was observed, also suggesting an effect of nitrifying organisms' growth and migration (Table 3.23, Figure 3.16). The results suggest that vermifiltration favours nitrite oxidation to nitrate, limited to certain maxima of circa 200 mg NO_3-N/L.

Table 3.22: Nitrate (NO_3-N) content and relative change as mean(±SE) in the vermifiltration unit of the combined system during period 3.

Time (days)	Feed (mg NO_3-N/L)	Effluent (mg NO_3-N/L)	Relative change (%)	*P*-value
0	143±(2)	154(±3)	+8(±3)	0.041
7	173(±1)	192(±1)	+11.0(±0.8)	<0.001
28	130(±3)	146(±7)	+12(±6)	0.10
35	147(±4)	141(±3)	–4(±3)	0.27
42	217(±5)	216(±5)	–1(±3)	0.84
49	205(±4)	205(±3)	0(±2)	0.98

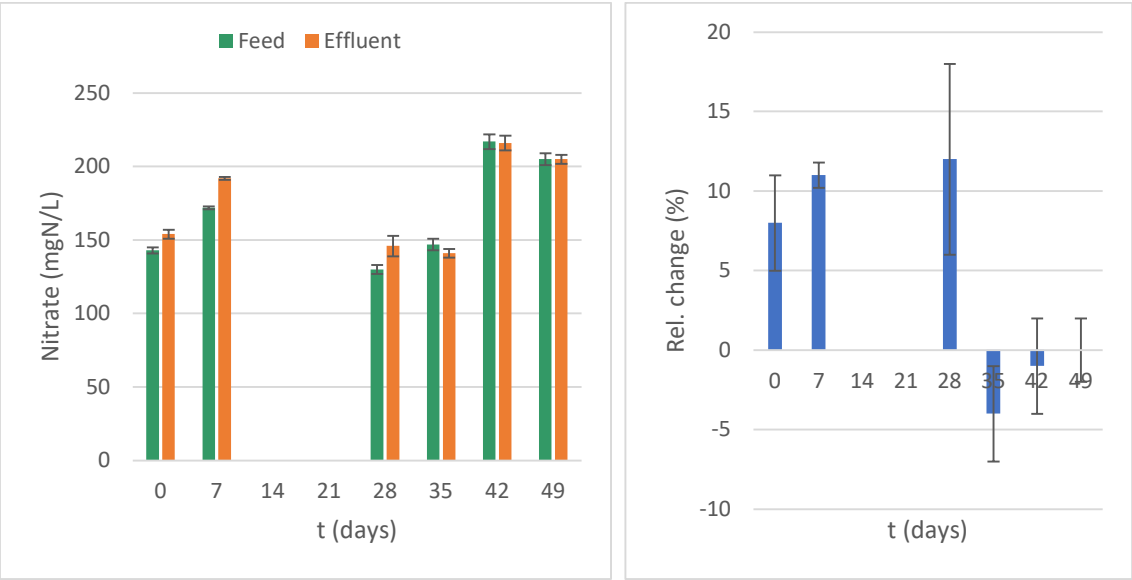

Figure 3.15: Nitrate (NO_3-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), over time during period 3. SE is represented by bars.

Table 3.23: Nitrate (NO_3-N) content and relative change as mean(±SE) in the vermifiltration unit of the combined system during period 4.

Time (days)	Feed (mg NO_3-N/L)	Effluent (mg NO_3-N/L)	Relative change (%)	*P*-value
18	27.2±(0.1)	97.3(±0.3)	+258(±1)	<0.001
25	45(±2)	80(±3)	+78(±8)	<0.001
32	70.4(±1.2)	118(±3)	+68(±5)	<0.001
39	111(±2)	123(±3)	+11(±3)	0.03

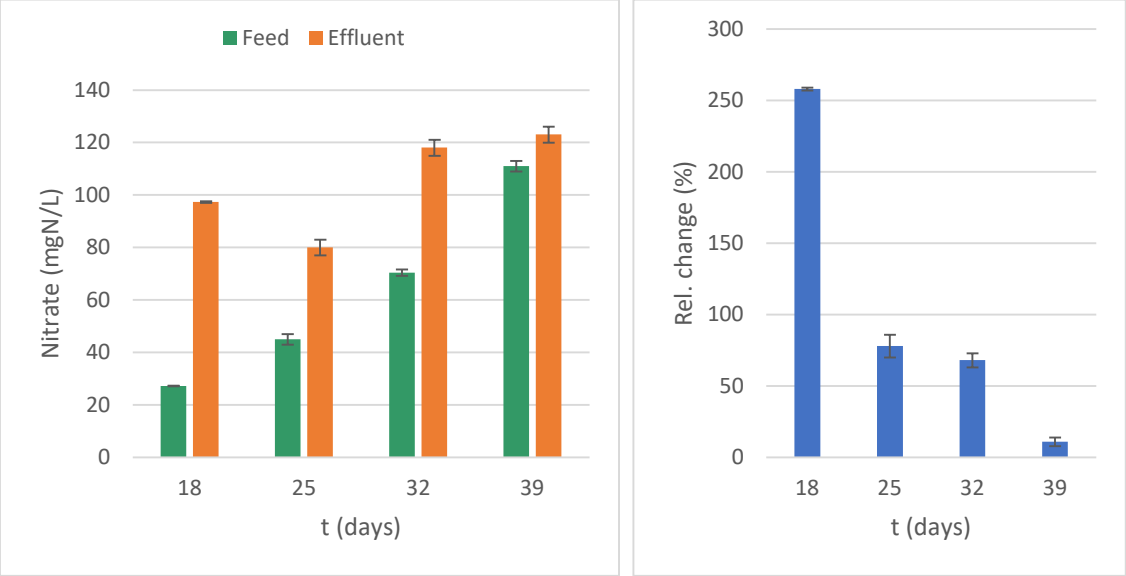

Figure 3.16: Nitrate (NO_3-N) concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

Nitrate averaged feed concentration, effluent concentration and relative change are shown in Table 3.24 and Figure 3.17 for period 3, and Table 3.25 and Figure 3.18 for period 4. Unaveraged values at each time point can be seen in Appendix III: Table III.5 and Table III.6. It was hypothesized that hydroponic treatment could remove nitrate as nutrient for plants. However, the relative changes ranged between positive and negative values, and at several points were not significant. The evolution of these data over time suggests a tendency for nitrate to stabilize around certain values, depending on the balance of all kinetics. The results do not suggest an efficient nitrate removal by hydroponic cultures in this sort of combined system, under the conditions of the present study. Final effluent had higher nitrate concentrations than the accepted values for discharge of wastewaters or recommended for irrigation purposes, according to Portuguese law (50 mg NO_3/L, corresponding to 11 mg NO_3-N/L) (Ministério do Ambiente, 1998).

Table 3.24: Nitrate (NO_3-N) content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 3.

Time (days)	Feed, three dates weighted average (mg NO_3-N/L)	Effluent (mg NO_3-N/L)	Relative change (%)	*P*-value
14	220(±11)	206(±20)	+13(±10)	0.35
21	210(±20)	204(±1)	–4(±32)	0.58
28	208(±13)	153(±1)	–27(±3)	<0.001
35	163(±15)	167(±3)	+2(±33)	0.65
42	146(±8)	203(±6)	+39(±3)	<0.001
49	194(±10)	203(±10)	+5(±7)	0.50

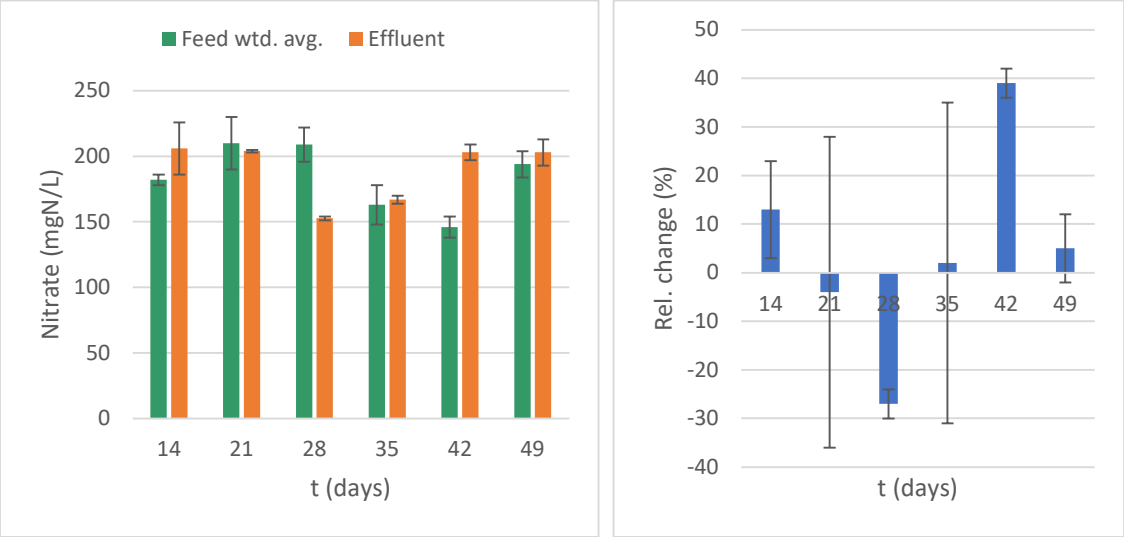

Figure 3.17: Nitrate (NO_3-N) concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 3. SE is represented by bars.

Table 3.25: Nitrate (NO_3-N) content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mg NO_3-N/L)	Effluent (mg NO_3-N/L)	Relative change (%)	*P*-value
25	76.8(±0.8)	74.0(±1.2)	–4(±2)	0.13
32	87(±5)	87.7(±1.3)	+1(±6)	0.81
39	107(±6)	110(±1)	+2(±6)	0.52

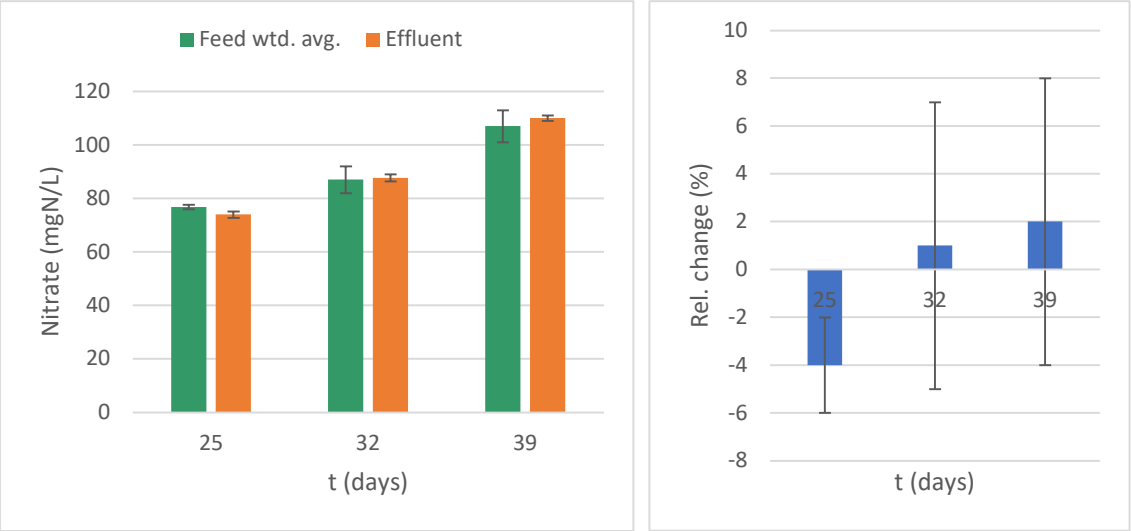

Figure 3.18: Nitrate (NO_3-N) concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

For a more complete understanding of nitrogen changes due to hydroponic treatment, relative changes in total nitrogen content were also calculated during period 4. The results from vermifiltration treatment (Table 3.26, Figure 3.19) showed small, not highly significant relative decreases in total nitrogen, which may be due to either biological accumulation by different organisms or denitrification. It should be noticed that, although total nitrogen was quantified in unfiltered samples, that did not include worms, plants or micro-organisms that remained somehow retained in the system. The hydroponic unit (Table 3.27, Figure 3.20; unaveraged values in Appendix III, Table III.7) at first contributed to increase and later stabilized TN content. Total nitrogen concentrations in the final effluent were higher than the maximum values legally accepted for wastewater discharge in Portugal, of 15 mgN/L (Ministério do Ambiente, 1997, 1998).

Table 3.26: Total nitrogen content and relative change as mean(±SE) in the vermifiltration unit of the combined system during period 4.

Time (days)	Feed (mgN/L)	Effluent (mgN/L)	Relative change (%)	*P*-value
18	106±(2)	98(±2)	–7(±3)	0.06
25	149(±2)	134(±6)	–10(±4)	0.08
32	160(±4)	144(±5)	–10(±4)	0.08
39	178(±6)	164(±4)	–7(±4)	0.14

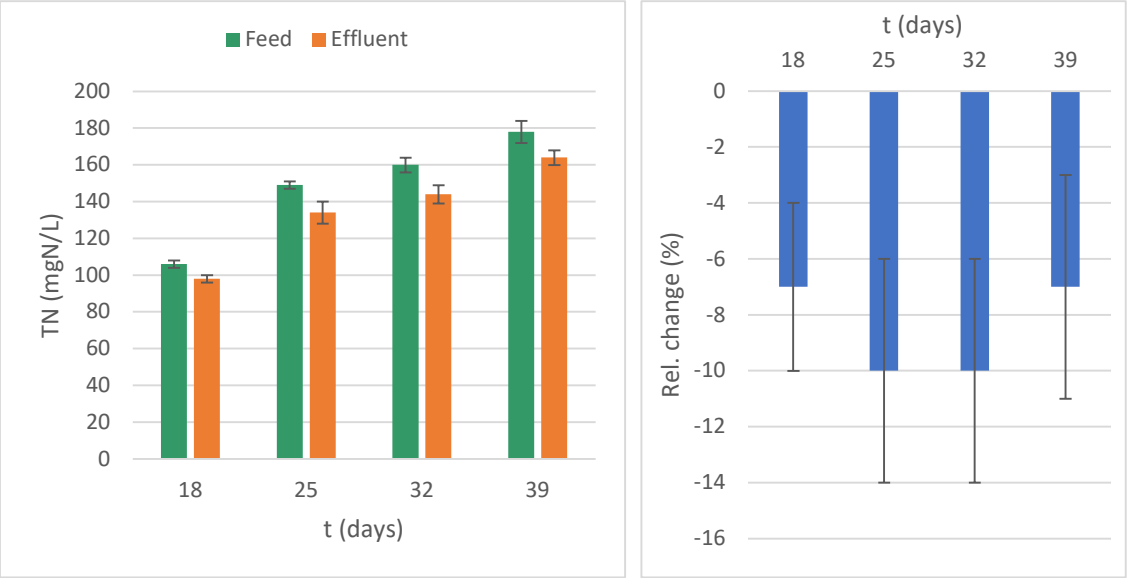

Figure 3.19: Total nitrogen concentration in the vermifilter feed and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

Table 3.27: Total nitrogen content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mgN/L)	Effluent (mgN/L)	Relative change (%)	*P*-value
25	83(±5)	105(±6)	+26(±10)	0.06
32	124(±12)	120(±3)	–4(±10)	0.55
39	142(±12)	140(±4)	–2(±9)	0.76

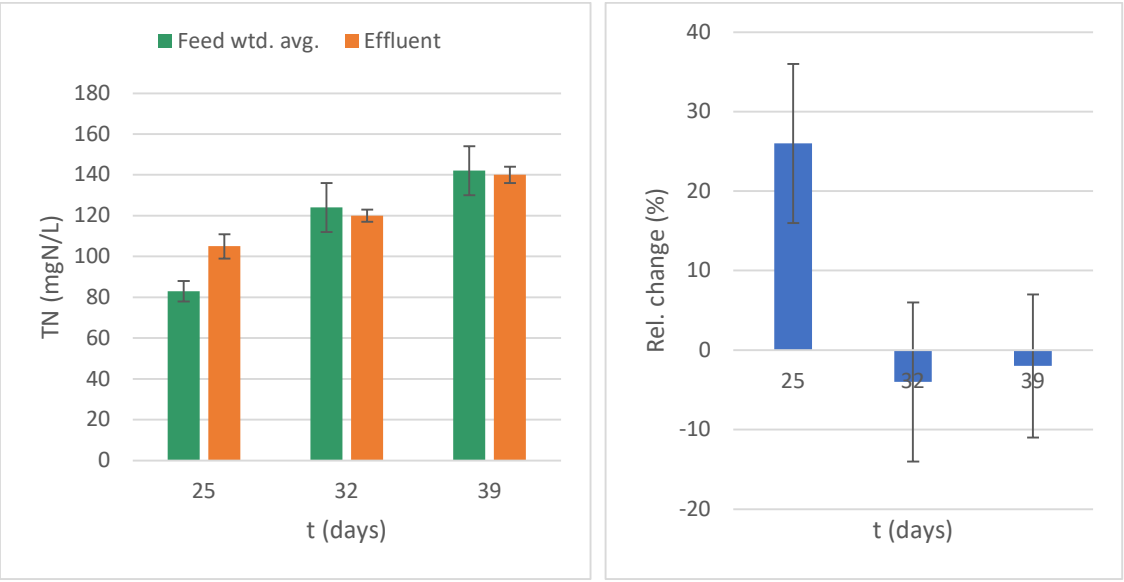

Figure 3.20: Total nitrogen concentration in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

Phosphorus

Total and dissolved phosphorus was analysed in the hydroponic feed and effluent in order to assess the removal capacity by such a treatment. To assess the difference between total and dissolved phosphorus in the same samples at each point, ANOVA significance tests were performed. The unaveraged values obtained in the feed and effluent over time can be seen in Appendix II (Table III.8, Table III.9, Table III.10 and Table III.11). It was found that generally total phosphorus content was slightly but significantly higher than dissolved phosphorus both in the feed and in the effluent. Around the same time in both periods, at 27 and 25 days respectively, no significant difference was found in the effluent (P = 0.12 and 0.71). At 34 and 32 days, on the contrary, no significant difference between total and dissolved phosphorus was found in the feed (P = 0.35 in both periods), which can be assigned in part to the effect of effluent recirculation. Phosphorus can be converted between dissolved and undissolved forms, both organic and inorganic, by numerous processes including biological uptake and excretion and chemical conversions (Vanni, 2002; Yeoman et al., 1988; W. Zhang et al., 2018). Also, a certain group of micro-organisms, called phosphate-accumulating organisms (PAO), are known to accumulate phosphorus as polyphosphates under aerobic conditions and release phosphate anaerobically (Seviour et al., 2003); if such PAO were present in the system, transitions from undissolved to dissolved phosphorus and back could be in part due to their activity. Explanation of the observed phenomena would require a more thorough phosphorus metabolism study.

During period 3, both total (Table 3.28, Figure 3.21) and dissolved phosphorus (Table 3.30, Figure 3.23) showed significant decrease as estimated by the applied method, suggesting that hydroponic systems can be successfully used to remove phosphorus from wastewaters. During period 4, decrease was observed up to a certain moment, when both total and dissolved phosphorus increased slightly (Table 3.29 and Figure 3.22; Table 3.31 and Figure 3.24). This may have been related to the worse overall resistance of planted radicchio compared to the cabbage, as was referred earlier, which possibly reflected on phosphorus uptake capacity. Nevertheless, hydroponic cultivation seems to be a promising technique of phosphorus removal from wastewaters, provided that sufficiently resistant plants are used and better conditions are created.

Table 3.28: Total phosphorus content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 3.

Time (days)	Feed, three dates weighted average (mgP/L)	Effluent (mgP/L)	Relative change (%)	*P*-value
42	39.2(±0.9)	20.9(±0.4)	–47(±3)	<0.001
49	33(±1)	24.3(±0.1)	–27(±3)	<0.001

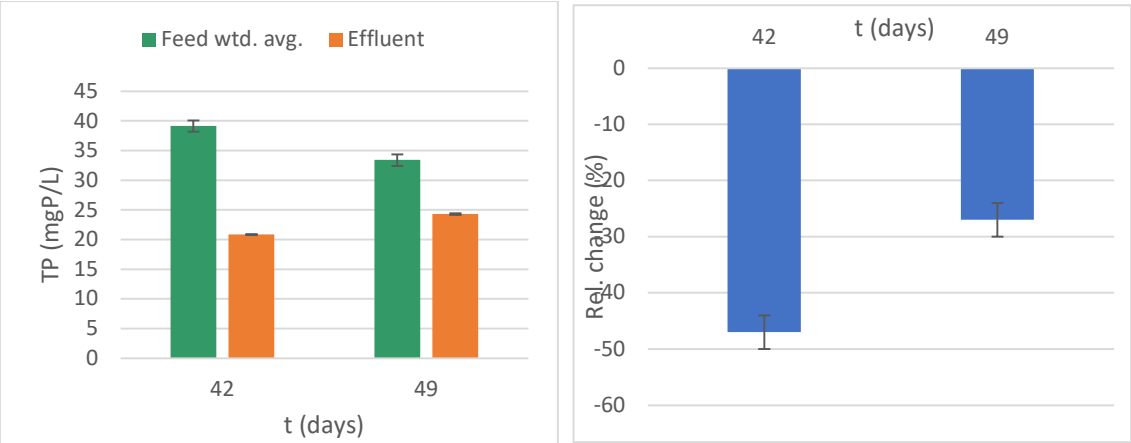

Figure 3.21: Total phosphorus content in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 3. SE is represented by bars.

Table 3.29: Total phosphorus content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mgP/L)	Effluent (mgP/L)	Relative change (%)	*P*-value
25	14.8(±0.2)	6.65(±0.05)	–55.0(±1.7)	<0.001
32	10.9(±0.3)	7.08(±0.04)	–35(±2)	<0.001
39	8.9(±0.3)	9.39(±0.06)	+6(±3)	0.013

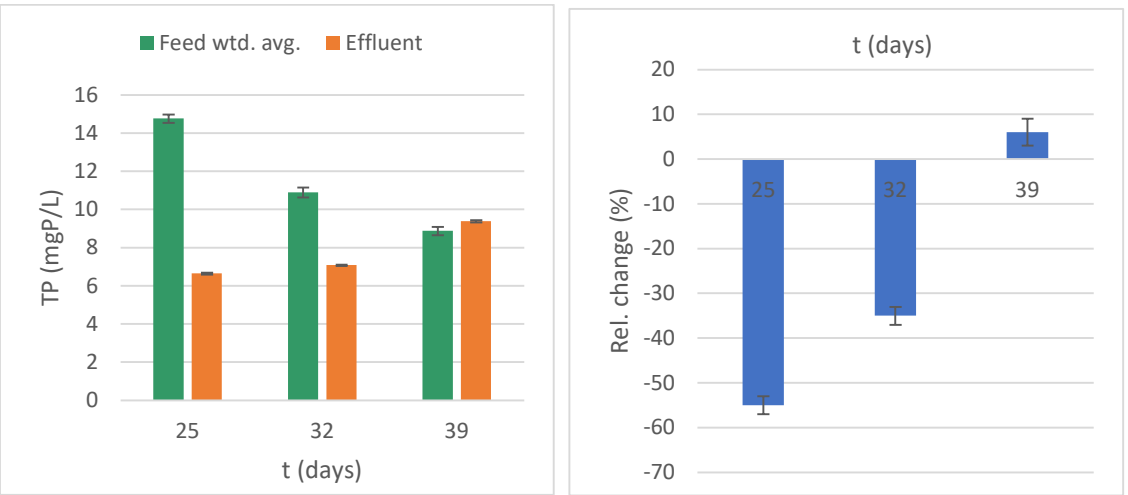

Figure 3.22: Total phosphorus content in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

Table 3.30: Dissolved phosphorus content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 3.

Time (days)	Feed, three dates weighted average (mgP/L)	Effluent (mgP/L)	Relative change (%)	*P*-value
42	33.2(±0.3)	18.5(±0.3)	–44(±1)	<0.001
49	35.3(±0.3)	22.4(±0.0)	–37(±1)	<0.001

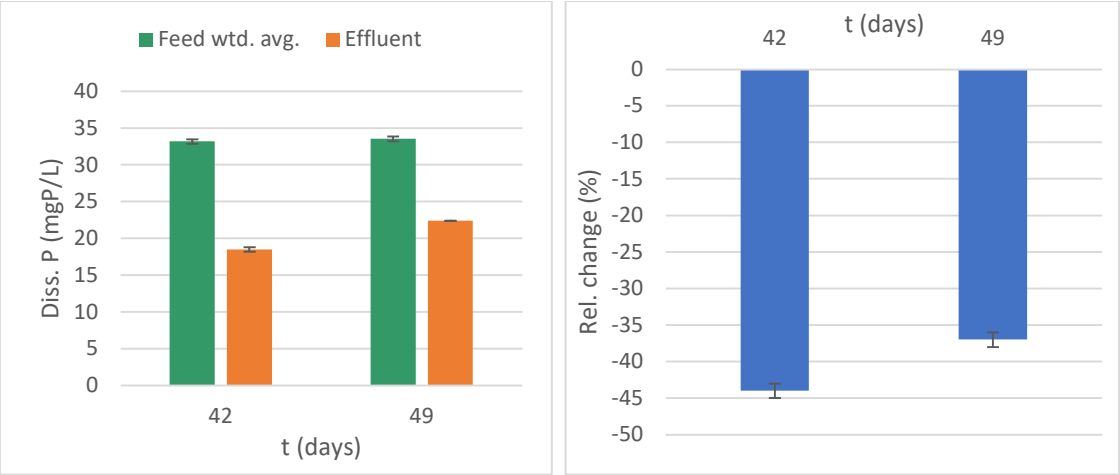

Figure 3.23: Dissolved phosphorus content in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 3. SE is represented by bars.

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Table 3.31: Dissolved phosphorus content and relative change as mean(±SE) in the hydroponic unit of the combined system during period 4.

Time (days)	Feed, three dates weighted average (mgP/L)	Effluent (mgP/L)	Relative change (%)	*P*-value
25	8.42(±0.14)	6.61(±0.08)	–22(±2)	<0.001
32	8.16(±0.05)	6.84(±0.03)	–16(±1)	<0.001
39	8.72(±0.06)	8.92(±0.06)	+2.3(±1.0)	0.05

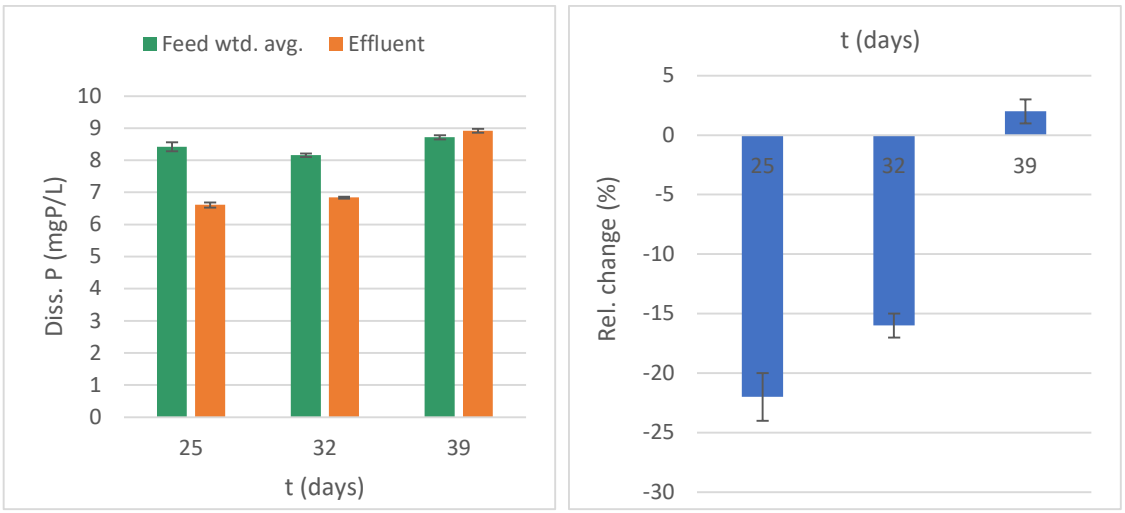

Figure 3.24: Dissolved phosphorus content in the hydroponic feed (weighted average of three measurements) and effluent, and relative change, as mean(±SE), over time during period 4. SE is represented by bars.

3.4 Problems and limitations found in the studied system

Throughout the period of study, the maintenance of the system and the performed analyses allowed to identify several important limitations that should be addressed to ensure a proper functioning of similar systems. In this section, the problems and proposed improvement measures will be discussed.

3.4.1 Feed composition

Piggery wastewater. As was revealed by the performed analyses, as well as visual observation of the obtained piggery wastewater samples, their composition and properties could vary wildly from one harvest to the next. Solids content and granulometry, colour and odour were observed to be different at different harvest times; different forms of nitrogen and phosphorus content was also hardly predictable. This was expected, considering that harvesting campaigns were not synchronized with any sort of operations at the piggery. Different procedures may have been carried out within the piggery facilities before wastewater discharge into the lagoons; wastewater flow into the lagoon system and therefore renovation rate could be variable; since the lagoons were of the open-air type, different times of exposure to different weather conditions (sunlight, temperature, precipitation) before harvest must also play a significant role. To control the composition of wastewater entering the vermifiltration-hydroponic system, previous analysis and adjustment to defined values of some parameters should be performed. Such parameters should be, at least, pH, electrical conductivity, and dissolved oxygen, since these could be measured quickly with a multiparameter probe and could already provide some indications on organic matter content, toxicity (ammonia, sulphide, toxic organic substances), and nitrification capacity. Turbidity measurements would point at TSS content and alert to the risk of feed line and vermifilter clogging.

Mineralization. Another problem is the electrical conductivity increase. Since the studied system involves recirculation of most effluent volume and there is significant organic matter mineralization activity in the vermifilter, as has been shown before and was confirmed in the present study, it was also observed that the electrical conductivity increases over time. This poses a threat to earthworms, hydroponically growing plants and bacterial communities, as all organisms have a certain range of tolerated salinity.

Plant nutrition. Hydroponically growing plants require liquid media with a specific nutrient composition, such as the well-known Hoagland solution (Hoagland & Arnon, 1950) for a healthy

growth and a good nutritious value as food. Feeding hydroponic growth with effluent from vermifiltration of animal wastewater in a recirculating system brings up serious challenges concerning a healthy medium composition for plant growth. Some essential nutrients may be absent from such a medium, and others are easily present in wrong amounts. To mitigate this problem, ideally the wastewater should be analysed regularly for important nutrients and corrected as necessary, the ideal supplementation being set up continuously. That might be very difficult to achieve on practice. This is likely an inevitable consequence of the hydroponic plants' intended double role as both water-treating organisms and source of food. It becomes apparent that making the crops play both roles in a satisfactory way might be one of the hardest challenges.

In conclusion, to address the above problems, the feed entering the vermifilters and, separately, hydroponic units, should be adjusted to a certain optimal composition. Feed correction measures could involve dilution adjustments, pH correction, addition of certain solutes and solids removal by mechanical devices to reach a certain optimized state. Feed mixing tanks and vermifilters should be sheltered from the weather; for the hydroponic units, a climate-controlled greenhouse could be advisable.

3.4.2 Solids accumulation

Regardless of the necessity to reduce feed solids content, it should not be attempted to remove the undissolved solids from the feed altogether since they are part of the components ingested by the earthworms and are removed by vermifiltration. These solids could become a problem over time, causing clogging of water lines and difficulties to pump operation. Water lines should, thus, be cleaned out and renovated periodically to ensure a smooth constant operation.

3.4.3 Biofilm formation

Microbial biofilms will be part of solid matter generated during the treatment process. These biofilms are important for the treatment since they retain active bacterial communities within the system; excessive biofilm growth, on the other hand, could contribute to system clogging, notably affecting water flow through the biofilter. It could also lead to limitations in oxygen supply to some spots, promoting local anaerobiosis with production of toxic substances. Obstruction of aeration system pores could easily occur, reducing bulk oxygen concentration

and further affecting aerobiosis. Some excess biofilm removal routines should be implemented, although the right way to do that would be the subject of a whole other study.

3.4.4 Biological contaminants

As was seen, the system was not able to remove coliforms; partial removal by vermifiltration was compensated by proliferation in the hydroponic unit. Some method of biological decontamination, such as ultraviolet light, could help solve this problem. Since both the earthworms and plants benefit from interactions with a variety of micro-organisms, a UV light source, if used, should be preferably placed at the final treated water discharge point.

3.4.5 Lighting

Another potential problem arising from the design and installation of the system was the absence of controlled lighting. The system was installed in a laboratory and operated under indirect variable intensity natural light from the existing windows. The use of lamps of appropriate spectrum and intensity would have allowed to provide the plants with a constant light and to establish a controlled daily photoperiod, all optimized for the best growth and metabolism. The choice to use natural light was based on the desire to design and test the simplest possible sort of vermifiltration-hydroponic system that could be upscaled for use on small swine facilities.

4. Real-scale treatment systems for swine farms

4.1 Theoretical representation

In order to propose an upscaled version of the studied system, it is useful to represent its components as bioreactors according to some known standard models.

The vermifilter module of the system can be treated as an immobilized biomass packed-bed reactor (Bailey & Ollis, 1986) with respect to the retained organisms: worms, the bacteria living in the worms' gut, and the bacteria growing on solid surfaces within the WVC layer. Although the worms are mobile and keep stirring the solids, their motion velocity is negligible compared to nutrient diffusion. For a more thorough theoretical description, considering the way it was constructed, the vermifilter should be described as a sequence of reactors, the first one being the WVC mixture containing worms, followed by several layers of different size inert materials where worms were not prevalent. Each earthworm, together with its gut bacteria, can be treated as a catalyst-carrying particle. The different types of solid particles (wood chips, vermicompost particles, sand and gravel of different sizes) allow adhesion of bacterial cells and thus act as catalyst-carrying particles, where the amount of catalyst per volume depends on each particle's available specific area (available surface area / volume). Adsorption-desorption equilibria between each particle and the liquid phase should be taken into consideration for a more complete theoretical modelling.

For the removed organisms – bacteria existing in the feed, the ones being excreted by the worms and transferred to the liquid phase, and the bacteria desorbed from the substrate granules – this part of the vermifilter is closer to a plug-flow bioreactor model (Bailey & Ollis, 1986).

Overall, vermifiltration treatment efficiency will depend the HRT as long as sufficient nutrient supply and low feed toxicity to different organisms are ensured. Positive correlations of treatment efficiency with HRT have been reported for different manure treatments (Bi et al., 2020; Marañón et al., 2001; Thy et al., 2003) and, on the other hand, for wastewater treatment by vermifiltration (Singh et al., 2019).

In the hydroponic plant cultivation module, like in the vermifilter, there were organisms that were retained, and others removed. The former included the plants and bacterial biofilm formed on different surfaces; the latter were the micro-organisms in the liquid phase. The hydroponic container was filled with liquid and homogenized by aeration, and therefore was closest in its design to a bubble-column reactor. These reactors are typically cylindrical and elongated in the vertical direction, but in the present case a wide horizontal area was important to accommodate the plants; the depth was chosen to limit the liquid volume while allowing plant roots to grow.

The aerator was constructed to provide good coverage of the whole horizontal area and an intense aeration flow to ensure a sufficient gas-liquid mass transfer; air bubble size is also an important factor for mass transfer efficiency (Bailey & Ollis, 1986).

For the hydroponic unit the usual homogeneous tank reactor mass balances for the substrates, products and microbial and plant biomasses can be considered (Bailey & Ollis, 1986), assuming an efficient homogenization by aeration. In such a unit, there will be influx of microbial biomass with the feed and its removal with the effluent. Plants will be introduced as seedlings and removed by harvest, which can be modelled as a continuous process if both operations are performed frequently enough.

The hydroponic treatment unit introduced plants as a new type of organism, different from both micro-organisms and earthworms. Higher plants are multicellular organisms with different types of structures and a vascular nutrient transport system connecting them. In this system plant roots were in contact with water, while aerial structures such as stems and leaves were not; however, all plant structures grew on the provided nutrients due to vascular transport. A more detailed model should, possibly, account for total plant biomass growth in correlation with substrate use and product formation, and also represent plant roots as a growing fixation structure for different types of micro-organisms.

In the hydroponic unit, two aims are pursued: production of edible crops and removal of nitrogen and phosphorus from the feed. The HRT will be the main parameter governing the quasi-steady-state nitrate and phosphate concentrations and should be optimized to allow both a continuous plant growth and an acceptable effluent quality. In hydroponic wastewater treatment longer HRT was reported to allow better efficiency (Keeratiurai, 2013). A possible solution could be to keep sufficient nitrate and phosphate concentrations in this unit for good edible plant growth and introduce an additional non-edible plant unit for the removal of the remaining nitrogen and phosphorus.

4.2 Scale-up proposal

The above theoretical and practical considerations allow to project the studied pilot-scale system onto a larger scale, suitable for small and medium-size pig farms. The already existing holding lagoons could be used for dilution of piggery wastewater with treated water. Before transferring into these mixing pools, the raw pig slurry should be freed from most solids by sedimentation or other techniques; the resulting liquid wastewater could be then pumped at a controlled rate into the mixing pools. Treated water could also flow into those pools continuously, being mixed by mechanical stirring as was used on laboratory scale, or

alternatively by turbulence and/or aeration. This additional aeration at this point would aid the elimination of oxidizable toxic components.

Vermifiltration and hydroponic cultivation units should be preferably constructed below the mixing pools level to receive the mixed wastewater by gravity. The system should be built inside a greenhouse to ensure climate control and equipment housing. Vermifilters would need to have a large enough area to accept the overflow from the mixing pools but be only as deep as necessary for an effective treatment. The wastewater would flow from the mixing pools and distributed over the vermifiltration unit area through a network of pipes equipped with valves; in order to adapt to different hydraulic loads, the vermifilter could be fragmented into compartments, and wastewater distribution could be channelled to some or all of those compartments according to the needs. More than one level of vermifilters could be stacked as reported by Aira and coworkers (Aira & Domínguez, 2008) in order to improve efficiency. The vermifiltered wastewater, intended to serve as nutritious medium for hydroponic crops, should be monitored periodically for the necessary nutrients content to allow adjustments.

The hydroponic unit(s) could be placed below the vermifilters level to receive the wastewater by gravity and homogenized by aeration through a network of air distribution pipes, as described for the pilot-scale system. The hydroponic unit could also be divided into compartments, where different crops could be grown and their specific nutrition needs met by separate nutrient addition. Water exiting the hydroponic unit should then be mixed, for instance, by aeration, part of it returning, by gravity, into the mixing pool and the rest flowing into a holding pool before allowing it to leach into the soil after tests. Water quality should be monitored in all units on a regular basis.

An example diagram of such a treatment system is presented in Figure 4.1.

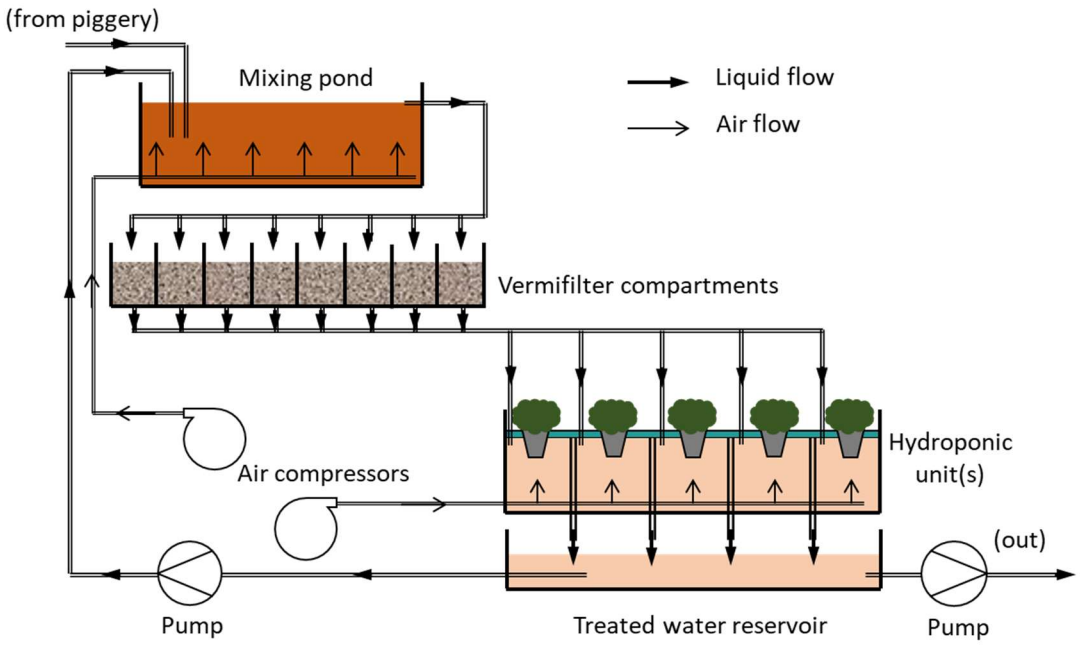

Figure 4.1: Diagram of proposed real piggery wastewater treatment system. Not to scale.

4.3 Sizing considerations

To size up a system as built in this work and discussed above to a real scale, real examples of pig farms can be taken. One example is a piggery of 3000 heads, reported to generate 20000 L of wastewater per day (Velho et al., 2012). At a 10% dilution this will mean a feed flow of 200000 L/day.

Since the vermifilter efficiency will largely depend on the volume of earthworm-inhabited layers, defining both the HRT and the total earthworm and associated microbial populations, and compost worms are restricted to a certain depth, then the parameter to be scaled will be essentially the vermifilter horizontal surface. The vermifilter used in this study had a surface of approximately 0.02 m^2, and the volumetric flow was on average 11 L/day. For similar efficiency, the upscaled surface area will then be:

$$A_{VF,up} = \frac{0.02\ \mathrm{m}^2 \times 200000}{11} \approx 360\ \mathrm{m}^2$$

This can be constructed, for example, as a horizontal rectangular structure of at least 40 m × 9 m; to make it 500 m^2, it could be 50 m × 10 m for better performance and eventual excess flow, or have any other shape suitable for the farm facilities.

For the hydroponic cultivation units, the efficiency will again depend on the total planted area, since plant biomass will be limited by that area. The hydroponic tray used in this study had a horizontal area of about 0.9 m^2. The upscaled horizontal area will then be:

$$A_{HP,up} = \frac{0.9\ \mathrm{m}^2 \times 200000}{11} \approx 16400\ \mathrm{m}^2$$

To make it approximately 20000 m^2, this can be constructed as a 140 m × 140 m square, a circle about 160 m in diameter or any other shape. This is comparable to the size of the wastewater holding lagoons typically used in pig farms, and a considerably large area to be occupied with a complex indoor water treatment facility. Since in the used pilot-scale system the hydroponic tray was only about 0.2 m deep, additional volume, and therefore HRT and increased efficiency, will be easily provided by a greater depth. In any case, such an area of built structure would imply a significant construction and maintenance cost.

An example of a small-scale swine farm found in the literature housed 20 animals, generating 1500 L of wastewater per day (Chao et al., 2008). This would correspond to 15000 L per day of diluted wastewater. Applying the above calculations to a piggery that size, the required areas would be:

$$A_{VF,up} = \frac{0.02\ \mathrm{m}^2 \times 15000}{11} \approx 27\ \mathrm{m}^2$$

This can be a 6 m × 4.5 m rectangle, which is the size of a relatively large room for a small-scale farm.

$$A_{HP,up} = \frac{0.9\ \mathrm{m}^2 \times 15000}{11} \approx 1230\ \mathrm{m}^2$$

This area could be a square of 35 m on each side, an oblong 123 m × 10 m rectangle or any more convenient shape. For such a small pig farm, additional constructed facilities this size are significantly larger than the pig housing space itself and could imply unbearable building costs. Projecting a larger scale system to be owned cooperatively or hired by multiple closely located swine farms to treat their wastewaters might prove more viable, paying off the investment if it generated profit through crop production, and the created jobs could make it a socially relevant project.

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5. Conclusions

A working pilot-scale system was built, combining vermifiltration with hydroponic crop cultivation for pig farm wastewater treatment. The system was fed continuously, and the treated wastewater was transferred back into the feed mixing unit in order to reduce water waste. Although challenging, this approach allowed to draw some useful conclusions.

Vermifiltration treatment was able to efficiently remove ammonia nitrogen, nitrite, BOD and suspended solids, and partially remove coliform bacteria from the pig farm wastewater; nitrogen increase was observed. Hydroponic treatment of vermifiltered wastewater was able to remove phosphorus but did not remove nitrate under the studied conditions. AOB and NOB were shown to be present in lower abundance in the liquid phase after vermifitration, suggesting that nitrification activity might be predominantly due to biofilm bacteria. A further decrease in BOD was observed, and coliforms increased after hydroponic treatment, probably unrelated to the growing plants; this can be viewed as a potential threat of hydroponic wastewater treatments. Nitrite and nitrate from the same piggery wastewater lot tended to stabilize at certain levels over time.

The proposed system aimed to treat piggery wastewater and produce edible crops at the same time. These are ambitious and somewhat conflicting goals. Efficient water treatment implies the removal of most pollutants, while good crop productivity needs excess nutrients to be present in the hydroponic medium. To ensure both, a very good knowledge of both the medium composition and the crops' needs is necessary.

Continuous systems with effluent recirculation and long hydraulic residence times create additional complications for the assessment of treatment efficiency. An easier way would be to depart from separate batch systems, which could be used independently for crop selection, nutrient media development and water quality analysis.

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6. Future work

Further studies could involve a continuous vermifilter/hydroponic system with a good knowledge of the best conditions for the selected crops. It will be essential to start with separate batch vermicomposting and hydroponic systems in order to ensure a better control and optimization. Both the liquid and solid phase composition should be analysed and adjusted as necessary to keep all the key parameters as constant as possible. This concerns all the important nutrients, toxic substances, biological contaminants, pH, electrical conductivity and solids. Water sampling and analysis for treatment efficiency assessment would need to be performed more frequently to provide quasi-continuous charts over time. The right physical conditions such as appropriate lighting by artificial light sources with a controlled photoperiod are also to be provided. In parallel, crop selection and adaptation studies must also be carried out.

After the batch systems are optimized, continuous treated water recirculation can be implemented. Constant controlled flows throughout the system will be necessary at that point. To prevent clogging, the control and removal of major solids should be ensured, and vermifilter and water lines should be cleaned periodically. Biofilm build-up should also be controlled in the hydroponic units, where it can obstruct the aeration pores and create local anaerobic conditions.

The implementation of similar systems on real scale will bring up further challenges. The efficiency of a similar water treatment system depends on the liquid flow and the volume of each compartment. Slower flows and larger volumes mean longer hydraulic residence times, increasing conversion efficiency. On the other hand, slow flows mean little throughput and could be useless in real pig farms. Large reactor volumes imply more space usage and higher costs of installation, power for pumps, aeration, stirring and automation, maintenance of all structures and equipment. Qualified personnel will be needed in any case, but especially in large-scale facilities.

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Appendices

I. Hydroponic nutrient mixture

Table I.1: Composition of the nutrient mixture used for hydroponic tray water supplementation in period 4, based on Hoagland nr. 1 solution (Hoagland & Arnon, 1950).

Component	**100 × *C* (mol/L)**	**C_{final} (mol/L)**
K_2SO_4	$2{,}50\times10^{-1}$	$2{,}50\times10^{-3}$
$MgSO_4$	$1{,}00\times10^{-1}$	$1{,}00\times10^{-3}$
$CaCO_3$	$1{,}00\times10^{-1}$	$1{,}00\times10^{-3}$
$CaCl_2$	$1{,}50\times10^{-1}$	$1{,}50\times10^{-3}$
H_3BO_3	$4{,}62\times10^{-3}$	$4{,}62\times10^{-5}$
$MnCl_2$	$9{,}15\times10^{-4}$	$9{,}15\times10^{-6}$
$ZnSO_4$	$7{,}65\times10^{-5}$	$7{,}65\times10^{-7}$
$CuSO_4$	$3{,}20\times10^{-5}$	$3{,}20\times10^{-7}$
Na_2MoO_4	$4{,}96\times10^{-5}$	$4{,}96\times10^{-7}$
$FeCl_2$	$4{,}00\times10^{-4}$	$4{,}00\times10^{-6}$
H_4EDTA or Na_2H_2EDTA	$4{,}00\times10^{-4}$	$4{,}00\times10^{-6}$

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II. Swine farm raw wastewater characteristics

Table II.1: Characteristics of raw wastewater from the swine farm used in this work (Pereira et al., 2019).

Parameter	Value
TDS	3100 mg/L
TSS	1900 mg/L
EC	0.9000 S/m
pH	8.00
COD	1997 mgO_2/L
BOD_5	149 mgO_2/L
NH_3-N	574 mg/L
NO_2-N	0.034 mg/L
NO_3-N	1.50 mg/L
TP	34.0 mg/L
PO_4-P	159 mg/L

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III. Parameters analysed in the combined vermifiltration-hydroponic treatment system.

Biochemical oxygen demand

Table III.1: BOD_5 in the combined system, period 3, as mean(±SE).

Time (days)	VF effluent/HP feed (mgO_2/L)	HP effluent (mgO_2/L)
28	5.4(±0.4)	1.64(±0.09)
35	5.9(±0.3)	1.83(±0.06)
42	5.8(±0.3)	3.01(±0.09)
49	6.1(±0.3)	2.17(±0.08)

Nitrogen

Table III.2: Ammonia content in the combined system (hydroponic unit), period 4, as mean(±SE).

Time (days)	VF effluent/HP feed ($mgNH_3$-N/L)	HP effluent ($mgNH_3$-N/L)
11	13.6(±0.0)	--------
18	0.090(±0.002)	0.057(±0.007)
25	0.0958(±0.0011)	0.064(±0.003)
32	0.181(±0.011)	0.103(±0.001)
39	0.124(±0.02)	0.099(±0.006)

Table III.3: Nitrite content in the combined system (hydroponic unit), period 3, as mean(±SE). LOQ: limit of quantification.

Time (days)	VF effluent/HP feed ($mgNO_2$-N/L)	HP effluent ($mgNO_2$-N/L)
7	0.0507(±0.0005)	<LOQ
28	0.0307(±0.0002)	0.0203(±0.0001)
35	0.0342(±0.0006)	<LOQ
42	0.0273(±0.0004)	0.0112(±0.0012)
49	0.0676(±0.0004)	0.0208(±0.0001)

Table III.4: Nitrite content in the combined system (hydroponic unit), period 4, as mean(±SE).

Time (days)	VF effluent/HP feed ($mgNO_2$-N/L)	HP effluent ($mgNO_2$-N/L)
11	8.98(±0.18)	--------
18	1.83(±0.03)	0.0399(±0.0001)
25	0.0898(±0.0003)	0.0192(±0.0001)
32	0.0856(±0.0003)	0.0374(±0.0001)
39	0.0660(±0.0001)	0.0397(±0.0001)

Table III.5: Nitrate content in the combined system (hydroponic unit), period 3, as mean(±SE).

Time (days)	VF effluent/HP feed ($mgNO_3$-N/L)	HP effluent ($mgNO_3$-N/L)
0	154(±3)	---------
7	192(±1)	166(±2)
14	220(±11)	206(±20)
21	208(±4)	204(±1)
28	146(±7)	153(±1)
35	141(±3)	167(±3)
42	216(±5)	203(±6)
48	205(±3)	203(±10)

Table III.6: Nitrate content in the combined system (hydroponic unit), period 4, as mean(±SE).

Time (days)	VF effluent/HP feed ($mgNO_3$-N/L)	HP effluent ($mgNO_3$-N/L)
11	28.3(±0.4)	---------
18	97.3(±0.3)	48.2(±1.6)
25	80(±3)	74.0(±1.2)
32	118(±3)	87.7(±1.3)
39	123(±3)	110(±1)

Table III.7: TN content in the combined system (hydroponic unit), period 4, as mean(±SE).

Time (days)	VF effluent/HP feed (mgN/L)	HP effluent (mgN/L)
11	39.7(±1.2)	---------
18	98(±2)	66.3(±0.3)
25	134(±6)	105(±6)
32	144(±5)	120(±3)
39	164(±4)	140(±4)

Phosphorus

Table III.8: Total and dissolved phosphorus content in the combined system (hydroponic unit), period 3, as mean(±SE).

	Total P		Dissolved P	
Time (days)	HP Feed (mgP/L)	HP Effluent (mgP/L)	HP Feed (mgP/L)	HP Effluent (mgP/L)
14	24.0(±0.2)	24.4(±0.1)	23.0(±0.1)	21.7 (±0.1)
28	49.3(±0.4)	19.4(±0.1)	26.7(±0.1)	19.6(±0.1)
35	35.2(±0.4)	22.9(±0.1)	35.7(±0.2)	18.0(±0.3)
42	33.3(±0.4)	20.9(±0.4)	36.4(±0.2)	18.5(±0.3)
49	23.8(±0.2)	24.3(±0.1)	22.1(±0.1)	22.4(±0.0)

Table III.9: Total and dissolved phosphorus content in the combined system (hydroponic unit), period 4, as mean(±SE).

	Total P		Dissolved P	
Time (days)	**HP Feed (mgP/L)**	**HP Effluent (mgP/L)**	**HP Feed (mgP/L)**	**HP Effluent (mgP/L)**
11	9.6(±0.2)	----------	8.71(±0.12)	----------
18	17.4(±0.1)	11.9(±0.0)	8.33(±0.04)	5.61(±0.02)
25	8.26(±0.12)	6.65(±0.05)	8.07(±0.02)	6.61(±0.08)
32	8.61(±0.12)	7.08(±0.04)	8.46(±0.03)	6.84(±0.03)
39	16.4(±0.1)	9.39(±0.06)	16.2(±0.1)	8.92(±0.06)

Table III.10: Total and dissolved phosphorus content comparison in the hydroponic unit, period 3.

Time (days)		**TP (mgP/L)**	**$P_{dissolved}$ (mgP/L)**	***P*-value**
14	**Feed**	24.0(±0.2)	23.0(±0.1)	0.023
	Effluent	24.4(±0.1)	21.7 (±0.1)	<0.001
28	**Feed**	49.3(±0.4)	26.7(±0.1)	<0.001
	Effluent	19.4 (±0.1)	19.6(±0.1)	0.12
35	**Feed**	35.2(±0.4)	35.7(±0.2)	0.35
	Effluent	22.9(±0.1)	18.0(±0.3)	<0.001
42	**Feed**	33.3(±0.4)	36.4(±0.2)	0.003
	Effluent	20.9(±0.4)	18.5(±0.3)	0.001
49	**Feed**	23.8(±0.2)	22.1(±0.1)	0.001
	Effluent	24.3(±0.1)	22.4(±0.0)	<0.001

Table III.11: Total and dissolved phosphorus content comparison in the hydroponic unit, period 4.

Time (days)		**TP (mgP/L)**	**$P_{dissolved}$ (mgP/L)**	***P*-value**
11	**Feed**	9.6(±0.2)	8.71(±0.12)	0.017
	Effluent	-------	-------	-------
18	**Feed**	17.4(±0.1)	8.33(±0.04)	<0.001
	Effluent	11.9(±0.0)	5.61(±0.02)	<0.001
25	**Feed**	8.26(±0.12)	8.07(±0.02)	0.20
	Effluent	6.65(±0.05)	6.61(±0.08)	0.71
32	**Feed**	8.61(±0.12)	8.46(±0.03)	0.35
	Effluent	7.08(±0.04)	6.84(±0.03)	0.008
39	**Feed**	16.4(±0.1)	16.2(±0.1)	0.065
	Effluent	9.39(±0.06)	8.92(±0.06)	0.005